

Public monopoly and private enterprise

The British government, no longer (one forgets) new, appears to have had a rush of blood to the head in the past few weeks in its reorganization of the public monopolies. With the British parliament well on the way to agreeing that the government should sell to the public a still undefined proportion of the shares in British Airways, it was flooded with a stream of proposals for the reorganization of other public monopolies. The British Post Office is to lose its monopoly on the carriage of letters. Three years from now, the telecommunications arm of the Post Office will no longer be the sole supplier of terminal equipment. To many people's surprise, the Central Electricity Generating Board is to lose its monopoly on the manufacture and sale of electricity, while British Rail is to divest itself of some of the largely peripheral activities which it has accumulated over the decades — the operation of docks and ferry services but also of hotels and golf courses. With such a flood of announcements, many British taxpayers must have been fearing that their government was about to break up the monopoly of the Inland Revenue by reverting to the ancient practice of putting out to open tender the right to collect taxes.

In reality, of course, the explanation of this fever of activity is more mundane. It has taken the British government a year or so to put flesh on the bones of its declared intention of exposing the public monopolies to more effective competition. With the summer parliamentary recess about to begin, ministers are trying to clear their desks and also to stake a claim on parliamentary time for legislation in the next session, beginning in November. The hope that it would be possible to deal with the public monopolies more quickly, and in such a way as to reduce the government's need to borrow from the money markets in the current financial year, much cherished a year ago, has now faded — with some exceptions, assets cannot be sold in a hurry except at a loss. The danger now is not so much that the British government will give potential purchasers of public assets too good a bargain at the taxpayers' expense but that by dealing piecemeal with the problem of public monopoly will arrive at a patchwork of decisions which are self-contradictory, even mutually nugatory.

But why not do away with the public monopolies altogether? This is what some intemperate souls are inclined to ask. A little reflection will show that such a radical solution, however attractive it may seem, is entirely impractical. Public monopolies of one kind or another will persist for as long as modern states persist. The clinching example is that of sewage disposal in built-up areas such as cities. Nobody in his senses would suggest that the provision of such a service should be left to private enterprise (as it used to be in the eighteenth century and is still in some rural areas). The essence of the case for local monopolies in sewage disposal is that the cost of providing the service is to a very large extent a capital cost, that the cost of two competing systems would be approximately twice the cost of one and that, if both were to remain viable, the users of the service would be required collectively to pay twice as much as would otherwise be necessary. (Ironically, in Britain, local monopolies for sewage disposal were uncontentious until recently, when the service was administered by elected local authorities; their transfer to the newly constituted water authorities has raised all kinds of doubts about the basis on which the service should be charged for.)

The difficulties in which the independent railway systems of the United Kingdom found themselves at the height of the nineteenth century railway boom is still a memorable illustration of how competition may not always be beneficial. It is, however,

important that the case for a public monopoly is not always strictly economic. Most national post offices have been given a monopoly to deliver mail on the grounds that same-cost delivery of a piece of mail to any address serves a valuable purpose in holding the community together.

At least in modern times, monopoly rights imply monopoly obligations. Because a monopoly can in principle make its customers pay through the nose for whatever service it provides, steps must be taken to prevent abuse. Even where public monopolies are financed by private, not public, capital, control of some kind is necessary, whence the great bureaucracies such as the Federal Communications Commission with which Washington is littered. Where monopolies are financed with public money, as in Britain, the agency of control is usually a nationalized industry and, paradoxically, consumers often have much less explicit evidence that their interests are well cared for. Governments are all too ready to issue directives which further short-term political interests against the long-term interests of consumers. (The way in which successive British governments have forced British Airways to buy British equipment against its wishes are only the most easily remembered scandal of this kind.) The objective of the recent wave of change in Britain is allegedly to make the public monopolies more efficient. The difficulty, by no means confined to Britain, is how to measure efficiency.

The crop of decisions that assailed the British parliament last week is from this point of view a mixed bag. The British government's decision that a proportion of the shares in British Airways should be sold to private investors is partly a money-making operation, although the government has also promised that it will "get the Treasury off the airline's back". For the rest, it is relying on the fond belief that once private investors are represented on the board of the airline, everybody will be more vigilant in looking for a healthy economic return on capital, so that the operation of the airline as a whole will be by definition optimally efficient. That expectation is, of course, naive. The best that can be hoped for is that the airline will be as efficient as it can be with one hand tied behind its back. For in the international jungle of air transport, the monopoly rights that matter are not those of being allowed to run an airline but of being allowed to land so many times a week at this or that airport, and these rights are controlled by governments, either bilaterally or through the International Air Transport Association (IATA). If nothing is done to make this system less restrictive than at present, there is no prospect that the reorganized airline will be better able to make efficient use of the technology of air transport than the airline that now exists.

The continued neglect of technology by the other public monopolies is indeed the most serious cause of discontent with the new arrangements now in prospect. In the delivery of mails, for example, private operators are to be allowed to compete with the British Post Office only in those circumstances in which it is feasible for them to charge at least £1 for each letter handled. Unless Sir Keith Joseph, the minister responsible, has cynically calculated that the Post Office will soon be charging much the same for its ordinary mail, this change will not markedly increase the pressure on the rump of the service to use automatic sorting devices more efficiently, to wonder about electronic mail or to devise other ways of making sure that people receive their mail. Similarly, although it is high time that the iniquitous monopoly of the telecommunications part of the British Post Office on the supply of terminal equipment was ended, that will not by itself



ensure that the rump of the monopoly will not repeat the appalling errors of the past three decades — blowing hot and cold about electronic switching equipment and so on. Sir Keith Joseph, the minister responsible, has gone no further than the decision of the United States Supreme Court in 1968 (the “Carterfone decision”) that the American public (but privately financed) monopoly should accept other people’s equipment at the ends of its telephone lines. What is to happen, in Britain, when quite new kinds of organisations ask permission to set up special-purpose communications networks (say, for data transmission) which the public monopoly does not wish for the time being to provide? Again, on past form at least, people will have to do without the benefits of a technology that they wish to use.

The decision that the monopoly of the Central Electricity Board on the generation of electricity should be modified is, from this standpoint, the oddest of those now announced. In the old days, the publicly owned electricity utility used to prevent people generating electricity from windmills sited in their back yards, but has recently been more accommodating. The British Government has not yet explained just what advantages its new dispensation will provide. Presumably, however, it will be possible for large

manufacturing plants to be equipped with their own electrical generating plants and even to sell some of the electricity surplus to their needs to consumers nearby. (Some, no doubt, will also think of building nuclear power stations.) On the face of things, however, it is hard to conceive of circumstances in which the independent generation of electricity in bulk offers advantages over the service the national grid can provide.

The scale on which electricity users opt out of the public monopoly will therefore be a crude measure of the monopoly’s failure. But again, the right to opt out will not by itself ensure that the service as a whole is technically efficient.

These issues are important because the public monopolies are often closely involved with novel or advanced technology. Not only in Britain, they are also conspicuously less innovative than they might be. The modest relaxations of the monopoly rights now announced in Britain are welcome, but their consequences will be marginal. Yet some at least of the public monopolies are here to stay. The long-term interests of their customers require more radical ways than those now devised of ensuring that they are technically adventurous, neither dogs in the manger nor stick-in-the-muds.

Subject in search of discipline?

How should the education of scientists be broadened? And how should intending scientists be helped to understand the wider implications of their professions? These are questions with which teachers have been much concerned in recent years. Increasing numbers of them have taken to devising curricula that include elements labelled “social relations of science” and the like. In recent years, science courses in higher education have been the chief beneficiaries (if that is the word) of this enrichment, but now there are signs that the habit is also catching on in the schools. It is therefore worthwhile that the Nuffield Foundation should have commissioned Sir Alec Cairncross (a wily economist once the British Treasury’s chief adviser) to take a cool look at schemes for broadening the curriculum in this way which have been introduced in recent years in British higher education. His report, now published (and to be had from the Nuffield Foundation, Nuffield Lodge, Regent’s Park, London NW1), should give pause to all those who think that their students will be better scientists if they can be persuaded to attend courses on the ethics of radioactive waste disposal, the social responsibility of scientists and the moral implications of genetic manipulation.

It is now more than twenty years since British universities began flirting with this means of broadening the science curriculum. One of the first courses to be established (at the University of Manchester) remains both the most ambitious and the most interesting. It constitutes a serious attempt to provide an undergraduate course with roughly equal amounts of science (physics, chemistry, physiology, etc.) and other subjects (history of science, economic history and so on). The original hope was that it would yield graduates with a capacity to work either as scientists or more generally. There are some thirty graduates a year, but the course appears not to be at the centre of school-leavers’ attention, while the people who emerge from it, cultivated though they are, rarely choose to practise as scientists. They contribute, rather to the pitifully small pool of people who work in business, industry or administration who also know what science is about. At other British universities, the experience of the past few years has been more disappointing. Some of the more ambitious attempts to set up genuinely amalgamating courses have had to be abandoned. Where science courses are supplemented with a small amount of more general chat about the social relations of science, the students appear enthusiastic but the value of their marginal activities cannot be evaluated.

It is easy enough to make fun of these extra components in the science curriculum. Sometimes they are superficial. Sometimes they are downright wrong-headed. University science teachers in British universities are, however, far too quick to make these and other scornful points. Cairncross says laconically in his report

that “it is a great misfortune of English education that it leads to so early and final a decision between those who study science and those who do not. This is a huge understatement. Outside the United Kingdom, it may not be fully appreciated that students entering science courses are almost always people who have been for the preceding two years rehearsing at school the studies they will follow later, and that many of them have been preparing for this curious exercise for a further three years. (Cairncross’s use of the word “English” is deliberate, for the Scottish universities are more liberal in what they require of new entrants.) The result is that the majority of those who enter science degree courses have long since ceased to bother with language (their own as well as other people’s), with history and economics. Is it any wonder that complaints abound that new British graduates are tongue-tied or illiterate or just plainly ignorant of the social setting in which they will have to work? And in such circumstances, can it be right to demean the attempts that university teachers make, however inadequate they may be, to compensate for the deficiencies of the educational system of which they are only a part?”

The only long-term remedy must be a reorganization of the educational system as a whole, but especially at the transition between secondary schools and universities. More immediately, there is a need for ways of enriching science courses in higher education that will be more substantial and more valuable than those now in vogue. Much of what is now provided for students under this heading centres around the much talked of Promethean dilemma. Nuclear fission means nuclear reactors but also nuclear proliferation. Molecular genetics means cheaper ways of making interferon (perhaps) but also (possibly) ways of undermining the natural course of evolution. The list of topics that make grist for this mill is endless. As ingredients of serious teaching, however, they are topics replete with pedagogical problems. The information available is incomplete. Books do not exist. The capacity of students maimed by their school education to grapple with the formless issues raised by such questions is quite inadequate. Their teachers are all too often bent on grinding other axes — political, ideological or religious. The Cairncross report has drawn attention to the need for some enrichment of science courses but has also spelled out the limitations of what is at present being taught. Has not the time now come for more serious attempts to make good the deficiencies of the British educational system? Science students might usefully be helped to keep up with a foreign language, to learn more serious economics or to discover in reorganized teaching laboratories that science is not cut and dried, as the textbooks have it, but as full of intellectual conundrums as any study of the humanities.

Proliferation a test of nerve in Congress

Washington

United States non-proliferation policy is facing its toughest domestic test for almost a decade, as Congress decides whether or not to veto President Carter's decision to allow the further export of nuclear fuel to the Tarapur nuclear power plant in India. The Nuclear Regulatory Commission (NRC) has already ruled that the export of 38 tons of enriched uranium requested by India would violate the Nuclear Non-Proliferation Act of 1978 (NNPA). The act forbids the export of fuel or technology to countries which have not adopted international safeguards on all their nuclear facilities.

President Carter has since overruled the NRC on the grounds that denying India the fuel would seriously prejudice political and economic relationships with India.

The dispute has brought to a head not only strategic differences between Congress and the State Department but also tensions between the United States and India rampant ever since India exploded a nuclear device in 1974.

Mrs Gandhi's return to power last year, and her subsequent firm refusal to eschew the possibility of India developing its own nuclear weapons, put an end to hopes that agreement could be reached. Now the Administration is trying to persuade Congress that political tensions in South Asia, and the consequent advantage of remaining on good terms with India, should override more narrowly focused policies.

The Administration and its critics find themselves on opposite sides of a narrow dividing line. The NRC, for example, taking a strict interpretation of the NNPA, refused to grant an export licence partly on the grounds that the shipment of the fuel would take place more than two years after the NNPA had been signed, the grace period for compliance. But the Administration points out that the request had been received in 1977; that the delays were the result of US indecision for which India should not be penalized; and that subsequent licence requests will be treated differently.

Then there is the question of whether, if the export licence is granted, it will be used as a precedent by other countries — for example Argentina, Brazil, South Africa and Iraq — to claim exemption from the act. Administration critics are standing firm on this point. The Administration however disagrees.

Another question is whether denying the fuel to India would violate the 1963 agreement, under which the United States guaranteed to provide fuel supplies to Tarapur for 30 years and, if so, whether India would then feel free to ignore other restrictions in the agreement.

"If the US breaks the agreement, then India can do what it wants, including using

plutonium from the fuel rods to make nuclear weapons", Dr Betty Lall of the US State Department said at a meeting organized by the Arms Control Association in Washington last week. Others, however, argue that India has long been aware that the obligation of the United States to supply fuel is contingent on India's compliance with US licensing requirements, however these may change.

Strict non-proliferationists favour the big stick. "Congress should draw a clear line and say 'no' to further nuclear commerce with India until the Indian government accepts international safeguards on all its nuclear activities", said a statement issued last week by several public interest groups, including the Natural Resources Defense Council and the Union of Concerned Scientists. The Administration still prefers to use softer methods, arguing that in the long run non-proliferation objectives have a better chance of success through dialogue rather than confrontation.

In Congress, which can overturn the President's decision if both houses agree by a simple majority before 12 September, the issue is in the balance. In the House of Representatives, feelings against the export

licence are running high; a majority of members of the Foreign Affairs Committee, for example, have signed a statement calling the decision to grant the licence "a dangerous mistake".

In the Senate, there is also some strong opposition, headed by Senator John Glenn, chairman of the Government Affairs Committee's Energy and Nuclear Proliferation Subcommittee.

In an election year, however, political dynamics could prove crucial. A certain amount of the opposition seems to have been stimulated by a desire to embarrass the Administration. The House committee's statement opposing the licences, for example, was supported by two-thirds of the committee's Democrats, but three-quarters of its Republicans.

The Administration is at present trying to convince a sufficient number of Senate Democrats of the value of treating the licence as a special case, with a promise of further negotiation towards a definite outcome one way or the other. Given that both houses have to concur in any disapproval, the Senate's refusal to do so would be sufficient to allow the export to proceed.

David Dickson

Plagiarism strikes again

The spate of publications with which Dr E.A. Alsabti has scattered the scientific literature in the past few years now seems destined to be followed by a similar spate of retractions. The accompanying letter (see page 437) from Dr Akiro Shishido, Managing Editor of the *Japanese Journal of Medical Science and Biology (JJMSB)*, says that a notice will appear in the August issue of the journal explaining that the editorial board now considers that Alsabti's contribution to that journal describes research originally carried out by Dr Daniel Wierda and Dr T L Pazdernick, first published in the *European Journal of Cancer*.

It is not yet known how many of the 23 papers listed in *Index Medicus* under Dr Alsabti's name are likely also to fall under a cloud. It is, however, known that Alsabti's paper "Lymphocyte transformation in patients with breast cancer", published in the *Japanese Journal of Experimental Medicine* (49, 101; 1979) is virtually identical with "The effects of surgery on lymphocyte transformation in patients with breast cancer", published by Dr Sylvia M. Watkins in *Clinical and Experimental Immunology* (14, 69; 1973).

As in other papers published under his name, Dr Alsabti acknowledges his gratitude to His Royal Highness Crown Prince Hassan for "financial support and encouragement". To Dr Watkins's original list of references is added in the

pirated version the reference "Alsabti, E.A., Lymphocyte transformation in bladder carcinoma, *J. Clin. Hematol. Oncol.* (1978), in press".

Meanwhile, an apparently unrelated case of plagiarism involving another Jordanian scientist, has come to light. Dr K.O. Kutschke, of the Canadian National Research Council, who is Editor of the *Canadian Journal of Chemistry*, says that in February 1978 his journal received for publication a manuscript called "Influence of electric charges on the corrosion of metals" a paper previously published by a group of Swedish authors — J.A. Hedvall, Nils-Gosta Vannerberg and P.O. Blomqvist — in *Acta chemica scandinavica* (22, 363; 1968).

In that case, Dr Kutschke explained this week, the plagiarized manuscript was not accepted for publication because the list of references included one to the Swedish group whose work had otherwise been pirated. Only curiosity, Dr Kutschke explained, prompted him to look up the original publication, whereupon he recognized the identity of the two papers.

On that occasion, it appears, the Jordanian author (whose name is being withheld) gave an address at a university in the eastern United States, but had given the University of Amman as the location of his research. On complaint from Dr Kutschke, the visiting appointment of the scientist was terminated.

US budgets

End in sight?

Washington

"The best we can hope for is to try to salvage a disaster and just make it a very bad year." This is how one Washington lobbyist last week summed up the prospects for next year's research budget as Congress continued, at the President's request, to chip away at the budget.

Since the beginning of the year, the prospects for science have changed substantially. When President Carter delivered his original request to Congress in January, he proposed a 13 per cent growth for basic research, about 3 per cent above the anticipated rate of inflation.

In March, after what the President's Science Advisor Dr Frank Press has described as the "traumatic experience" of a review designed to cut public expenditure and balance the overall budget, the increase was reduced to 8 per cent, barely enough to keep pace with inflation. Now even this figure is looking optimistic.

As far as congressional authorization committees (which approve programmes for funding) are concerned, there have been relatively few problems in meeting the President's requests for cuts and in some cases even greater cuts have been made.

Both houses of Congress have agreed on an authorization bill for the National Aeronautics and Space Administration (NASA) which, at a total of \$5,600 million, is \$70 million above the revised request for the agency. The two houses agreed in particular to restore funding for some of the long-range support programmes that had been cut earlier.

Similarly the National Science Foundation has so far done relatively well. The House of Representatives and the Senate have passed very different authorization bills, with the latter incorporating provisions for a major "women in science" programme, and this could involve some redistribution of funds within the agency. But the total in each case is at or above the Administration's \$1,074 million request.

But in the appropriations committees — which decide how the federal budget is to be allocated — it is a different story. Most appropriations bills have still to be acted on; but in their determination to pare the federal budget wherever possible, several appropriations committees have taken basic research as one of their targets.

The worst affected so far has been the Department of Energy, which supports a large proportion of the physics programme. The department's request for energy research activities, trimmed slightly by authorization committees, was recommended for a 12 per cent cut.

This proposal stimulated intense lobbying by universities and research laboratories, as well as the Office of Science and Technology Policy, and when

the budget was debated on the floor of the House, an amendment proposed by Mr Don Fuqua, chairman of the House Science and Technology Committee, to restore most of this cut was agreed by a wide margin.

Furthermore the intense effort involved in protecting the energy research budget is being taken as an indicator of the strength with which scientists will increasingly have to fight to retain their share of the budget pie in other areas as well.

As far as other agencies are concerned, no action has yet been taken by Congress on the National Institutes of Health budget, which for the most part does not require separate authorization. Congress is unlikely to cut back on the Administration's request for a 5.2 per cent increase to \$3,350 million; but neither is it likely to award the generous increase of previous years.

Some concern is being expressed about the Administration's decision to cut back heavily on the number of training grants. These may be traded in committee against the proposal to maintain at 5,000 the number of new and competing investigator-initiated research grants.

The House has authorized a total of \$627 million for basic research in the Department of Defense, and the Senate has authorized a comparable figure. This is lower than the \$644 million in the Administration's revised budget request, but still represents a real growth of 8 per cent over 1980 funding, far higher than for any other research agency. Also, for the third year in a row, the House appropriations committee has proposed cutting \$30 million allocated to a competitive grants programme to the Department of Agriculture, in favour of support for programmed research support through designated land-grant colleges. As in previous years, this is likely to be partially restored in the Senate.

Despite protests that reducing the Environmental Protection Agency's research activities will restrict its effectiveness, the House has authorized a budget 3 per cent lower than the Administration had requested for research, from \$413 million to \$396 million.

David Dickson

International laboratory

Danes turn soft

Danish biologists are preparing to make a strong recommendation to their Minister of Education next month that Denmark should continue to belong to the European Molecular Biology Laboratory in Heidelberg. Their previous report on the question of continued membership of EMBL was lukewarm, and persuaded a government advisory committee to decide that Denmark should rescind the EMBL treaty from mid-1981 (*Nature*, 26 June).

That report, now in circulation, recommended that EMBL should increase the number of permanent scientific posts (there is at present only one) and have fewer short-term contracts (now about 50); there should be more detailed research plans than hitherto; and EMBL should submit its plans for the next five-year period (1985–90) to Denmark in time for a "well-founded" decision on membership.

Before then, says the report, the research councils should "consider whether it is desirable in principle that Denmark should participate in building up European laboratories in fields where the proportions of the problems and investment prevent attempts to establish national solutions or international collaboration in utilizing national laboratories" — thus implying that in fact EMBL was unnecessary.

EMBL's recruitment policy appears to be based in part on the assumption that the best molecular biologists are young, but the report concludes that these younger scientists require "a well-established environment with firm scientific traditions and with older experienced scientists" before they can achieve important results. Hence, the working party concluded, it is not possible to build up a good laboratory if nearly all the scientific staff are young postdoctoral fellows on short-term contracts.

Moreover "it must be expected that small working groups of scientists whose contracts are reviewed every three years will chiefly choose problems ensuring results within a short period, but on the other hand not offering great possibilities of all-important results".

The report also questioned the research management of EMBL, saying that "it does not appear very satisfactory that the individual working groups are not asked to submit fairly specific plans for their work and that the decision-making bodies, including among others the EMBL council, are given such a vague basis for making decisions". The research groups are also too small, the report considered, being no larger than those at national laboratories.

On the positive side, the report made special mention of the groups on data processing and the utilization of computers; this part of EMBL's activities was "very promising".

Nevertheless, it is felt strongly at EMBL that it is too soon to make a proper assessment of the laboratory's achievement. The laboratory was established in its present permanent headquarters in Heidelberg in 1978, and many of the staff were recruited then. The constitution of EMBL, modelled on that of the European sub-nuclear physics organization CERN, says that no person may apply for tenure until he or she has worked at EMBL for three years. Few have reached that position. The laboratory's director, Sir John Kendrew, says that there should be a marked increase in the number of tenured posts in a few years.

EMBL acts as a service centre, with P3 and P4 laboratories, outstations using synchrotron light at Hamburg and neutrons at Grenoble, and instrumentation and computer groups at Heidelberg. So it is necessary, EMBL thinking goes, for the staff scientists concerned to pursue the most up to date lines of research, to keep them in touch with the immediate needs of the scientific community. Thus flexibility is a prime goal, and short-term contracts and small groups provide it.

The Danish meeting on EMBL towards the end of next month will consist not only of biologists. There will be representatives of the research councils for natural science, agriculture and medicine, just as there were on the original working party that produced the original report, so it is difficult to predict the outcome. However, whereas that report recommended that the government should foot the £120,000 EMBL bill, the new report may suggest that a single research council takes the subscription into its own budget. An obstacle may be that at present no Danish biologist works at EMBL, although a computer scientist and some ancillary staff do so.

Robert Walgate

Scientific instruments

Nature spin-off

The colour filters supplied with the 3 April issue of *Nature* have turned out to have an unexpected use. In some laboratories, it seems that the filters are superior to others on the market in the contrast they provide.

Dr R. Zeki of University College London, whose article on colour vision prompted the distribution of the set of three colour filters (*Nature* 284, 412-421; 1980), said last week that he had received a number of comments on the value of the filters, which he had passed on to Polaroid, the manufacturers.

One of those who have found the red filter of particular value is Mr David Owen, a graduate student in the laboratory of Dr C. Harwood, of the University of Newcastle upon Tyne. One series of experiments is to identify extra-

chromosomal genetic elements in bacteria. The procedure is to separate DNA molecules by gel electrophoresis and to make molecules of different classes apparent by treating the gel with a fluorescent dye that binds to DNA.

Mr Owen explained last week that he had had some trouble finding a suitable filter for photographing the pattern of visible light stimulated by ultraviolet radiation, so he had tried "Dr Zeki's red filter".

"To our surprise", he said, "the resulting photographs were far superior... in that the DNA bands were clearly visible against a black background". The accompanying illustration is an example of such a photograph, in which the bright central band represents chromosomal DNA and the sidebands DNA from plasmids.

No agreed explanation of this result is available, although it seems likely that the narrowness of the transmission band of the red filter may account for the sharp contrast.

The Polarioid Corporation has apparently developed these filters largely for research purposes. A spokesman for the company said last week that filters with narrow transmission bands should be available from more conventional sources, but that the company might reconsider its decision that its filters would not be put on the market if the demand for them turned out to be sufficient.

High-energy physics

Trouble ahead

Washington

As if bleak budget prospects were not enough, high-energy physicists in the United States are facing another delicate question — what to do about continued difficulties in the development of superconducting magnets for the 400 × 400 GeV accelerator (ISABELLE) now under construction at the Brookhaven National Laboratory on Long Island.

The official line is that a substantial effort must be maintained to resolve the difficulties, even if this means cutting further into the laboratory's operating

funds for experiments and bringing in help from across the physics community.

But attention is now also being paid to what have been called "graceful failure modes". At best this could mean lowering the maximum energy of each of the two intersecting beams of protons from 400 to, say, 300 GeV; at worst, it could mean cutting losses and scrapping the whole project.

Given the continued risk of failure in the ambitious superconducting magnet programmes at Brookhaven and at Fermilab, some physicists are suggesting that there is a need to broaden the base of the whole nuclear physics programme — in particular, by diverting resources to electron-positron machines directly competitive with Europe's proposed LEP in Geneva.

ISABELLE is scheduled for completion in 1986, when it will be able to explore the physics of proton-proton interactions at energies an order of magnitude higher than those which can be reached on comparable existing machines.

It had always been anticipated that developing superconducting magnets to deliver the required high fields would be difficult. But given the success of early prototype magnets, nobody realized how great the difficulties were likely to be.

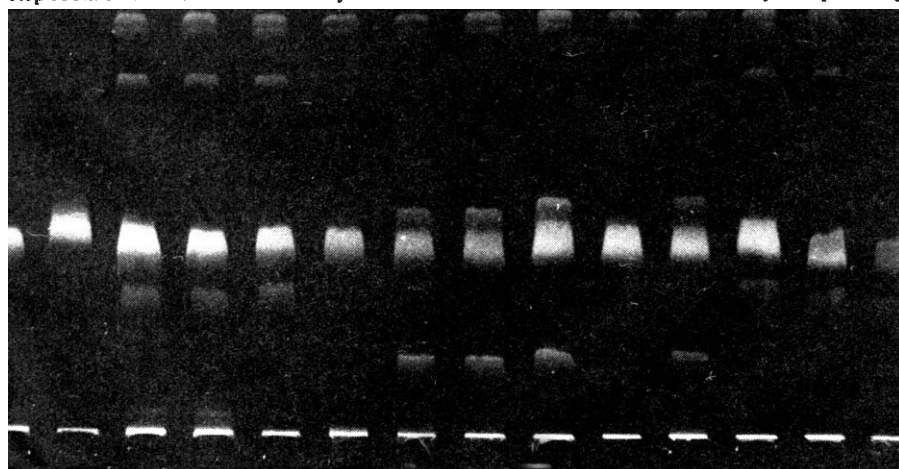
ISABELLE will use 1.7-m quadrupole and 5-m dipole magnets, the former operating with a gradient of 6 kG cm⁻¹, the latter at a field strength of 50 kG. So far the quadrupoles have operated with no difficulty. But major problems have arisen with the dipoles, which have not reached either the planned maximum fields or field quality — and of which more than 700 will eventually be required.

Brookhaven has mounted an intense research and development programme to pinpoint the difficulty. The number of people working on the magnets has been increased almost four-fold over the past two years. And outside help has also been sought from the Massachusetts Institute of Technology's magnet laboratory and the Lawrence Berkeley Laboratory.

The problem is one of engineering rather than theory — that of keeping the magnets in a superconducting mode while building up the required field. Although Brookhaven scientists are optimistic that an answer will eventually be found, they admit that they are still uncertain about precisely what is going wrong.

ISABELLE's difficulties were a major focus of attention when a sub-panel of the Department of Energy's High-Energy Physics Advisory Panel met in Woods Hole, Massachusetts, last month to report on the current status and prospects of US high-energy physics.

Considerable discussion took place about whether, in the light of the "serious and unexpected setbacks" to ISABELLE's construction programmes, the specifications for its performance should be revised downwards. If each beam only



operated in the 60 to 300 GeV range, it was argued, this would still be sufficient to produce a large amount of important physics, even without the possibility of investigating collision energies between 600 and 800 GeV.

In the end, the panel refrained from making any such formal proposal. But it did state that current information from Brookhaven was insufficient to assess whether the pilot production of the dipoles was likely to begin in mid-1981 as planned. "If it is not, the ISABELLE project will encounter significant delays", the sub-panel reported.

Brookhaven scientists have been heartened by the report's recommendation that the successful completion of ISABELLE — including any necessary R and D funding — should remain a top priority of the Department of Energy, as well as becoming "the responsibility of the entire national community".

At the same time, however, others are warning of the dangers of failure in what has become recognized, both at Brookhaven and to a lesser extent at Fermilab, as a high-risk/high-benefit programme still facing large engineering uncertainties.

"The US high-energy programme is now highly dependent on the successful solution of such engineering problems and I would recommend greater diversification to reduce that dependence", Dr Wolfgang Panofsky, director of the Stanford Linear Accelerator Centre (SLAC), told a meeting held in Washington last week to celebrate 50 years of particle accelerator construction.

SLAC is promoting its own proposal, developed by Dr Burton Richter, to construct a machine which would collide bunches of electrons and positrons, passed in tandem down the linear accelerator and then in separate directions around a ring at the end.

Dr Richter is pushing for early funding of the so-called Stanford Linear Collider (SLC), attempting to finesse experiments on CERN's LEP and claim discovery of the intermediate vector boson. The Woods Hole sub-panel examined the proposal closely, describing it as a "fascinating and challenging new departure in accelerator technology". However, the sub-panel refused to endorse SLC for immediate funding. In particular, questions were raised about whether SLC's predicted luminosity would be sufficient to make it truly competitive with LEP, which will also have a larger energy range.

David Dickson

Dead Sea

Coming to life

Theodore Herzl's dream of a canal from the Mediterranean to the Dead Sea is at last a cost-effective proposal, the Israeli government was informed last week. Use

of the 400 m drop between the two seas to generate hydroelectricity could now match the cheapest generation from fossil fuel stations, according to a government-appointed working party chaired by Professor Yuval Neeman of Tel Aviv University.

Assuming an initial investment of \$800 million, the canal with a cascade of hydroelectric installations would, said Dr Neeman, pay for itself in 30 years, simply from the 100–150 MW of hydroelectricity produced. In addition, the canal could provide the sites for nuclear power stations which cannot be accommodated along the crowded Mediterranean coast. (That between Ashkelon and Ashdod has been found to be earthquake-prone.) A thermal power station using the water from the canal but fired by local oil shale deposits could also be built at Ein Bokek.

Ecologically, the main benefit of the canal would be to replenish the waters of the Dead Sea. The only natural feeder of the Dead Sea is the lower Jordan, which draws its water from the Sea of Galilee and from its east bank tributary, the Yarmuk. The Sea of Galilee is however the source of water for virtually the whole country, while the Jordanians have almost completed a major irrigation project involving the diversion of the Yarmuk into the East Ghor Canal. When this is complete, the lower Jordan will shrink even further to a mere 200 million m³ annually, mostly return flow from irrigation.

Evaporation loss from the Dead Sea is 1,200 m³ a year, so that the sea has shrunk considerably — the S'dom potash works have become stranded and a special channel has been built to pump brine to the evaporation pans. By November 1978, in a Knesset debate on the proposed canal, both government and opposition were agreed that any further shrinkage could bring irreversible ecological damage.

Then came the drought of 1979. By September, the Dead Sea had split in two at the narrowest point (some 3 km across) and, according to Shlomo Drori of the Dead Sea Development Company, there was a dry plain some 12 km long between the northern part of the Sea and the southern fragment, then split into a number of pools. Meanwhile, plans were going ahead to use the Dead Sea for generating electricity on the "solar pond" principle, based on the temperature difference developed in brines exposed to sunlight where the density gradient is too great to allow convection mixing. The first solar pond power station was opened at Ein Bokek in December 1979.

The ecological advantages of the proposed canal are admitted by all concerned. Until now, they have been the paramount concern, with hydroelectricity considered rather as a valuable spin-off. As late as the Knesset debate of 1978 on the canal, Energy Minister Yitzhak Moda'i stressed that electricity could still be generated more cheaply from fossil fuels.

Professor Neeman's estimates, for the first time, reverse this situation. However, with the Israeli economy in a state of crisis — inflation is now running at 130 per cent per annum — it is by no means clear whether the government could raise the necessary \$800 million.

Vera Rich

Biogenetic hormones

Insulin trial

Eli Lilly, the US pharmaceutical company, has taken another step along the road to producing human insulin on a commercial scale. Last week, it announced a \$40 million programme to build two plants at its factories at Speke in the United Kingdom and Indianapolis in the United States for the commercial production of human insulin from genetically engineered bacteria. Ultimately, human insulin produced in this way should be much cheaper than the insulin currently extracted from pig and beef pancreas.

Full-scale production, however, is still a few years off. First, any problems cropping up with manufacture on a large scale will have to be resolved and extensive clinical trials have to be done. The first trials with insulin from the company's US laboratories began on healthy people earlier this month at Guy's Hospital in London. Initially at least, the trials are being conducted in the United Kingdom to avoid delay in getting permission from the Food and Drug Administration for trials in the United States.

The tests at Guy's Hospital mark the first time that any recombinant DNA hormone has been injected into humans. Preliminary tests to measure the effectiveness of the new insulin in reducing the blood glucose level of healthy people and their immune response to it should be complete in a few weeks. By the autumn, tests should begin on diabetics. It is expected that human insulin will be tolerated without the production of antibodies. Some diabetics do produce antibodies to insulin derived from pigs and cattle. One possible advantage of human insulin is that it will minimize or eliminate the risk to middle-aged diabetics of eye and liver damage.

The techniques Eli Lilly are using are those developed by Genentech (which will get a cut of the profits) whereby synthetic genes for the A and B insulin chains are put into an *Escherichia coli* plasmid. The bacteria expressing the two genes are fermented separately, killed and broken up to extract the A and B chains. The chains are then chemically combined to produce human insulin. So far, no method of getting genetically engineered bacteria to excrete their products has been found, but Eli Lilly claims that its method of extraction has produced pure insulin free from bacterial fragments.

The production of human insulin from recombinant DNA is more complicated

than the production of antibiotics, where bacteria excrete the product. Nevertheless, it should prove far less costly than the current methods of extracting porcine and beef insulin. The cost of human insulin to the consumer, however, will depend on its penetration into the market and competition from other companies. When costs will come down is still uncertain, especially since Lilly cannot even say yet when it will be in a position to start commercial production.

Judy Redfearn

Environmental protection

Bulgaria ahead

Bulgaria is to spend more than 1,000 million lev (£500 million) on environmental protection during the next five years, President Todor Zhivkov announced recently. He was addressing a meeting held to celebrate the 80th anniversary of the Bulgarian National Agricultural Union.

In Bulgaria, President Zhivkov said, the Constitution makes environmental protection a nationwide matter, dealt with by specially created bodies at all levels of state management. Ecological problems are a compulsory subject in higher educational establishments and throughout the educational system. Special organizations have been set up to deal with erosion and bring reclaimed or neglected soil back into use. Bulgaria, he said, is a world leader in reforestation, planting 400–500 decare per year.

Zhivkov unequivocally rejected the view of "bourgeois ideologists" who maintain that the violation of the established ecological balance is the result of the modern scientific-technological revolution. On the contrary, he said, this revolution is essentially "our greatest ally in harmonizing relations between society and nature". Although the published data on natural resources are so "very alarming", Zhivkov reiterated that "being aware of the danger is a signal for action, not a cause for pessimism".

Bulgaria is not rich in natural resources, and before the Second World War it was the most backward but one of the European countries (Albania ranked last). Now, however, Zhivkov claimed his country is a "developed industrial agrarian country", with an agriculture capable of feeding "one and a half Bulgarias" and a target of "two Bulgarias" in 1985. Referring to his African tour of two years ago, Zhivkov suggested that if Nigeria, Angola, Ethiopia and Mozambique could apply Bulgarian-type organization and technology to their agriculture, they too would be producing surpluses. Ethiopia, in particular, he said, has especially good soil which could not only support the present population but also "feed a neighbouring state".

Vera Rich

One journal disowns plagiarism

SIR — Your recent article "An Outbreak of Piracy in the Literature" makes it apparent that the *Japanese Journal of Medical Science and Biology (JJMSB)* was a victim of an outbreak of paper piracy by a person called Dr A. Alsabti. I am writing this letter on behalf of the Editorial Board of *JJMSB* to make our position clear. Our unfortunate experience shows how difficult it is to be aware of dastardly and minutely planned piracy of papers.

We first received this manuscript entitled "Effect of Platinum Compounds on Murine Lymphocyte Mitogenesis" on 20 November 1978 from the stated address — Department of Developmental Therapeutics, M.D. Anderson Hospital and Tumor Institute, University of Texas System Cancer Center. After careful reviewing, the manuscript was sent back on 25 December 1978 with referees' comments to Dr Alsabti at his changed address (7520 Brompton Blvd, No. 736, Houston, Texas 77025). The revised manuscript was received in our editorial office on 16 January 1979, but it was still not satisfactory for publication and it was again sent back to him on 12 February. The final revision was received on 2 March, and the editorial board accepted it for publication on 12 April 1979. On 11 May 1979, a galley proof was sent to him and he asked us to send any further correspondence to his new address — 7511 Teal Run, Houston, Texas 77071. On 11 June 1979 the paper was finally published in the April issue of our journal (*Japan. J. med. Sci. Biol.* 32, 53–65, 1979).

The editing of Dr Alsabti's paper therefore followed quite a normal course. However, it may be important to add here that we received three other manuscripts from him at around the same time. These were:

- (1) Effects of tumor cell culture supernatants from various tumor cell line and normal tissues on macrophages.
- (2) Tumor dormancy established in DBA/2 mice with P815Y mastocytoma cell lines.
- (3) Tumor dormant state established in DBA/2 mice by sinecomitant immunization and challenge with lymphoma cells: Role of endogenous viruses in its mechanism.

All these papers were rejected.

Almost one year later we received a shocking letter from Dr D. Wierda of the Chemical Industry Institute of Toxicology, North Carolina, USA. His letter told us an almost unbelievable fact, that the paper entitled "Suppression of Spleen Lymphocyte Mitogenesis in Mice Injected with Platinum Compounds" contributed by himself and Dr T.L. Pazdernik to the *European Journal of Cancer* (15, 1013–1023, 1979) is identical to Dr Alsabti's "Effect of Platinum Compounds on Murine Lymphocyte Mitogenesis"

published in our journal. Dr Wierda enclosed copies of proofs demonstrating the authenticity of his work and suspected that their manuscript was probably pirated during the process of review for the *European Journal of Cancer*. He added that their manuscript was received on 10 October 1978, accepted for publication on 5 January 1979 and published in the August 1979 issue of *European Journal of Cancer*.

In urgent response to Dr Wierda's letter I wrote to Dr Alsabti on 9 June 1980 to ask for an explanation of this unusual situation, specifying a time limit of 15 July, 1980. This letter was sent to his last corresponding address. I also asked Dr Freireich, head of the Department of Developmental Therapeutics, M.D. Anderson Hospital and Tumor Institute, to forward my letter to Dr Alsabti.

On 30 June 1980 Dr Freireich wrote to tell me that he could not forward my letter to Dr Alsabti as his address was unknown. However, he proved that Dr Alsabti had never conducted any scientific research worthy of publication, either in the laboratory or clinic, during his 7 months' stay in his department, and suggested further that his large number of publications are all either outright plagiarisms or were totally imaginary.

We have so far received no reply from Dr Alsabti, although the deadline we gave him has already expired. Furthermore, Dr Wierda notified me on 21 July of the article "Jordanian Denies He Pirated Papers" (in the 11 July issue of *Science*) in which Dr Alsabti claimed that he had not published the paper in *JJMSB*. We therefore decided to retract Dr Alsabti's paper to protect the characters of Drs D. Wierda and T.L. Pazdernik. *Japanese Journal of Medical Science and Biology* hereby announces retraction of his paper from our journal. Our "Retraction Notice" will appear in the coming August issue of *Japan. J. med. Sci. Biol.* 33 (4), 1980 as follows:

The Editorial Board of the Japanese Journal of Medical Sciences and Biology here announces retraction of the following paper from the Journal:

"Effect of Platinum Compounds on Murine Lymphocyte Mitogenesis" by E.A.K. Alsabti, O.N. Ghalib, and M.H. Salem. *Japan. J. med. Sci. Biol.* 32(2), 53–65, 1979.

Judging from several lines of evidence (1–5), this work must have been authentically carried out by Drs D. Wierda, and T.L. Pazdernik, whose paper was almost simultaneously published in the *Eur. J. Cancer* (15 (8), 1013–1023, 1979) under the title of "Suppression of Spleen Lymphocyte Mitogenesis in Mice Injected with Platinum Compounds".

1. Letters from Drs D. Wierda & T.L. Pazdernik.
2. Correspondence to the Head of Department of Developmental Therapeutics, M.D. Anderson Hospital and Tumor Institute, the University of Texas System Cancer Center, with which Dr. Alsabti was affiliated.
3. *Nature* 285, 429–430 (1980).
4. *Science* 208, 1438–1440 (1980).
5. *Science* 209, 249 (1980).

Akira Shishido

National Institute of Health, Tokyo, Japan.

CORRESPONDENCE

Biologists mis-cite

SIR,—The importance of checking original sources is well recognized, but doing so becomes harder as the literature grows. I describe here an attempt at quantification of the reliability of citations.

Since familiarity with the cited literature is essential, I selected 28 of my own, not faultless, publications and 136 related publications by other authors. These deal mostly with snail physiology and with ionic and acid-base regulation in animals.

Errors found include misquotation of quantitative data, confusion of hypothesis with fact, of crustaceans with molluscs, of one species with another and of experimental variations with natural variations; also invention, simplification and statements contrary to what was demonstrated. Along with 33 such errors occurring in 29 citing publications are 11 instances of correct information being used with a wrong reference, or with none. Some of the correct citations were almost too simple to be capable of being wrong (such as "Recent investigations include that of. . ."). Such citations can be useful, but at least 12 citing publications include apparently pointless references to the cited publications or, more important, omit others known to the authors that are clearly of greater relevance.

There are 3 instances of references being given in the text, but not in the bibliography, and 4 mistakes of the opposite kind. Of the 205 entries in the 136 bibliographies, 14 contain mistakes that could hinder, though not prevent, the location of articles (wrong page number, year or publisher). Other, mostly trivial slips, including altered punctuation and omission of words or letters, occur in a further 55 references, sometimes several in each. 58 of the bibliographies contain at least one error. Most errors are in titles, which, in accordance with editorial policies, many of the other references do not include.

Fifty-six per cent of the citing publications contained at least one of the above faults. Especially in bibliographies, slips must often be due to hasty note-taking before citation is specifically envisaged; the original lack of care may then be forgotten during preparation of the manuscript. Blanchard¹, in a letter since misrepresented in print, has described (I think) how he found that, of 6 references to a particular paper in chemistry, 4 contained errors in volume or page number. The assessment concerning the appropriateness of some of the citations is subjective, but is a reminder that other factors may influence an author's choice of citations than the interests of the reader^{2,3}.

R.F. BURTON

*Institute of Physiology
University of Glasgow
Glasgow G12 8QQ, UK*

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1. Blanchard, D.C. *Science* **185**, 1003 (1974).
2. May, K.O. *Science* **156**, 890-892 (1967).
3. Garfield, E. *Current Contents, Life Sci.* **23**, 5-9 (1980).

Chemical warfare

SIR,—Your leading article of 10 July makes the point that verification of an agreement to ban chemical weapons, which was never easy, becomes even more difficult in relation to production of the components of binary chemical munitions. An alternative approach, assuming that an agreement had been reached, might be to monitor the stockpiling and/or issue of protective clothing (especially gas masks) for military or civilian use, and any

military exercises in such clothing. If the major powers had entered into the agreement in good faith there would be no need for clothing capable of protecting against nerve agents, and certainly not of military exercises by troops wearing it. It is very unlikely that a country intending to use chemical weapons could do so without making preparations to protect its own personnel on a scale which would escape detection by the intelligence organizations of the other countries which were party to the agreement. From an agreed date, after sufficient countries had ratified the agreement, all clothing for protection against nerve gases would go into bonded stores, and any military exercises involving such clothing would be forbidden.

If the argument is valid, such an indirect monitoring should suffice to detect any intention to infringe the agreement — even though the most important factor remains confidence that in no country do chemical weapons serve its people's real interests.

J.H. HUMPHREY

*17 Mortimer Crescent
London NW6 5NP, UK*

Budapest disclaimer

SIR,—Your article by Vera Rich (5 June) refers to an interview with me at the Budapest International Fair. Having studied the contents of the article, I must state with the utmost regret that it lacks the desired accuracy. I therefore feel it appropriate to correct the article at some points.

At the beginning of our talk I conveyed to Miss Rich that as the Hungarian Academy of Sciences was not a participant at the 1980 Budapest International Fair, academic research was not being presented at this event. I explained that at the exhibition, in accordance with Hungarian science policy, attention was drawn to scientific results serving practical benefits and achieved at universities and other institutes of higher education within the sphere of the Ministry of Education. Furthermore, I emphasized that great effort has been and is still exercised by the Ministry of Education towards putting research results into practice as soon as possible, thus promoting progress in our country. Universities agree with us in setting these goals, as was confirmed by the fair. I continued by emphasizing the importance of the demand made by users in connection with practical research, but I did not say "We make what foreigners want to buy".

In the course of the ensuing discussion, Miss Rich raised the question of how our scientists react to research being directed towards practical purposes. In answer, I said that the great majority seems to exercise the necessary goodwill in this respect but that not everybody is fit to fulfill the demands required by practical research.

In this sense, the statement attributed to me in the article that I do not agree with the pragmatic orientation of our research policy is incorrect; what I said was that the intention of the authorities of giving a pragmatic orientation to research policy causes some of our scientists anxiety. Accordingly I cannot accept, being myself also an advocate of emphasizing the need for practical research, the statement attributed to me that "It is a pragmatic solution to direct more scientists to industry and not good for research".

I do not know which facts led Miss Rich to the conclusion that we place less emphasis on the socialist virtue of the development of scientific research in Hungary. I am convinced

that this could only be a misunderstanding.

GYÖRGY PÁRIS

*23 Szerb Street
H-1056 Budapest
Hungary*

VERA RICH writes: I should feel most distressed if misunderstandings cloud Mr Paris's recollections of what I remember as a most friendly and open conversation. Although the Academy of Sciences was not, however, exhibiting this year, the pavilion was clearly marked on the official plan as housing, *inter alia*, the Academy. If, because of interpretation difficulties, I have inadvertently confused views that Mr Paris put forward on his own behalf with views he quoted from scientists who disagree with him, I would wish to point out, in tendering him my apologies, that it is by no means common to meet an official spokesman who is ready both to put his own view and to quote his opponents.

Adjectival concerns

SIR,—I suggest that we generalize the convention implicit in the naming of the Earth's year. We suffer from a surfeit of planetary adjectives — should one speak of the mercuric or mercurial year, the saturnian, saturnine or saturnalian? Can one, in a reputable journal, mention the venerale or uranal year? How much simpler to name the planetary years *ymar*, *yven*, *year*, *ymar*, *yjup*, *ysat*, *yura*, *ynep* and *yplu*! How fortunate that *y* is both vowel and consonant!

PETER RASTALL

*Department of Physics
University of British Columbia
Vancouver, British Columbia
Canada V6T 1W5*

Unsplit infinitives

Dr Glascock from Reading's an erudite man,
Full of wit and grammatical zeal;
In *Nature*¹ this May, he asked for a ban
On the customary scientists' spiel.

Speak active, not passive, Dr Glascock
advised;
Attach participles where they belong.
Above all be sure that you are appraised
Of how syntax matters in song.

Alas (and alack), as they say in some places,
The verses we liked but we shortened the
missive.
One way and another, the result was red faces;
Nature had split one of Glascock's infinitives!

Much worse was to come, to readers' chagrin.
Good Garton noticed the error²
And promptly blamed Glascock, not *Nature*,
for sin.
What anger, what shock and what horror!

Nature now tenders both Garston and
Glascock
Regrets for its error and shame;
What verbs should be split is for authors to
say
It is we who shoulder the blame.

The moral's quite clear, as we know too well.
The jargon leaves much to desire.
But do not rely upon rhymed doggerel
And to Belloc do not aspire.

Editor, *Nature*

1. *Nature* 8 May p.66.
2. *Nature* 12 June p.434.

NEWS AND VIEWS

Solar size variation

from David W. Hughes

Is the sun getting bigger, getting smaller or staying about the same size? Astronomers are divided as to the answer, some say it is shrinking, some say it remains constant.

John A. Eddy, of the High Altitude Observatory, Boulder, Colorado and Aram A. Boornazian, a mathematician with S. Ross and Co., Boston favour the shrinking Sun, and talk in terms of a contraction of about 0.1% per century. Their results were published in *The American Astronomical Society Bulletin* 11, 437; 1979. A recent paper by Irwin Shapiro of the Massachusetts Institute of Technology contains results which disagree with those of Eddy and Boornazian. Shapiro concludes that the change is less than 0.03% per century.

Eddy and Boornazian used data obtained at the Royal Greenwich Observatory. At Greenwich the precise position of the centre of the Sun, with respect to the stars, was measured on every clear day at noon. The times at which the limbs of the Sun crossed a fixed meridian wire were recorded. Knowing the Earth's rotation rate and the Earth's orbit enables the time difference between the two crossings to be converted into a solar horizontal diameter. Values for this quantity are shown in Fig. 1.

The vertical solar diameter has also been measured since 1836 using a micrometer screw at the eyepiece. However inaccuracies in the mechanical screw and problems of atmospheric refraction make the vertical readings less accurate than the horizontal ones. The decrease in apparent Sun size as a function of time can be easily seen in the Greenwich data.

Sun transit measurements from the Naval Observatory, Washington, DC, were also analysed and the same decreasing trend was noticed. There was, though, a consistent difference between the two data sets, the Sun's horizontal diameter appearing to be 1 arc s larger from Washington, and the vertical diameter about 0.5 arc s smaller. (The Sun's diameter is about 1,917 arc s). This introduces one of the problems of the measuring technique. The results depend

to an extent on the atmospheric transmission, a quantity that varies from place to place on the globe. The judgement of the time when the limb crosses a meridian wire is effected by irradiation, a physiological effect produced by the contrast between a bright object (the Sun in this case) and a dim one (the sky background). The Greenwich data shows for example a consistent difference between the apparent diameter of the Sun in winter and summer.

The Greenwich results indicate that the horizontal diameter is diminishing by 2.25 arc s per century and the vertical one by 0.75 arc s per century. The fact that the latter result suffers from atmospheric refraction makes the authors cautious about claiming that the Sun is changing its shape.

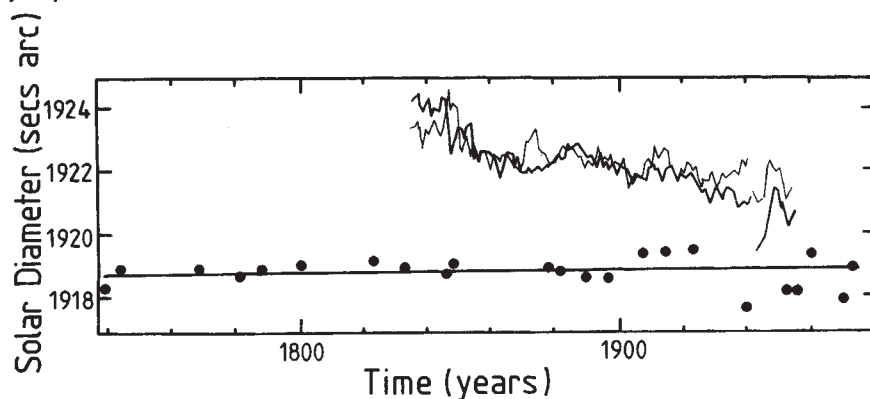
Now a constant shrinkage rate of 2.25 arc s per century for an object that is about 1,917 arc s in diameter means that the Sun shrinks to a point in 90,000 years time and was twice its present size 90,000 years ago. Both these are clearly ridiculous conclusions, the latter being completely unsupported by geological evidence, and Eddy and Boornazian are convinced that they are dealing with an oscillating Sun. The responsible agent could be thermal cycling in the convective zone or periodic variations in the convective efficiency, the period being hundreds of years, similar to the period between Maunder minima. The

Kelvin-Helmholtz time scale of the convective envelope, the time it takes a photon to escape assuming that the photons movement is not assisted by convective motion, is also of the order of hundreds of years.

Before theoretical speculation gets out of hand let us consider the results of a more recent analysis that indicates that the Sun has been the same size for the last 250 years. The solar diameter can be easily obtained from an analysis of the observations of the transits of the planet Mercury in front of the Sun. These transits occur about 13 times per century, in May and November, when Earth and Mercury are nearly aligned, and have been carefully observed since 1677. The observations consist simply of the times of external and internal contact. Mercury has an apparent diameter of 10 arc seconds (1/200 that of the Sun) and takes about 5 hours to cross the Sun. The observed times of contact are affected by seeing conditions and the 'boiling' of the Sun's limb and are often uncertain by more than ten seconds.

Irwin I. Shapiro has published his analysis of the transit data in a recent edition of *Science* 208, 51, 1980. His results are also shown in Fig. 1. The values obtained by the transit technique are free from any significant dependence on the variation in the rotation rate of the Earth and on our knowledge of the orbits of Earth and Mercury. The line through the

Fig. 1 The apparent diameters of the Sun as a function of time. Upper graphs show the annual averages obtained by Eddy and Boornazian from the Royal Greenwich Observatory data. (Thin line — vertical diameter, thick line — horizontal diameter). The dots represent the solar diameter as obtained from observations of the transit of Mercury by Shapiro.



David W. Hughes is in the Department of Physics, University of Sheffield.

data in Fig. 1 has a gradient of 0.05 ± 0.10 arc s per century indicating that the Sun is possibly expanding but also that a conclusion that the Sun is of a constant size is entirely in keeping with the quoted uncertainties.

This result supports the findings of Sofia, O'Keefe, Lesh and Endal (*Science* **204**, 1306, 1979) who imply that changes in the solar constant (a measure of the energy output of the Sun) are directly related to the solar size. From the constancy of the solar constant Sofia *et al* conclude that the rate of change of solar diameter must be less than 0.6 arc s per century.

How do we explain Eddy's result? Firstly the difference shown in Fig. 1 between the solar diameter measured by Mercury transits and the diameter measured by meridional transits is due to the irradiation effect caused by the solar disk — sky contrast. This can easily amount to the two

s or so difference shown in Fig. 1. This irradiation is a function of atmospheric transparency and decreases as the transparency decreases. Greenwich being a suburb of London is obviously affected by the growth of London's population and the atmospheric effects of the increasing coal consumption between 1836 and 1950. Eddy and Boornazian find, however, that an unrealistic transmission decrease of at least 20% is required to explain their results. Irradiation effects also depend on the instrument, and the observing technique and the observer. The latter can be ruled out as over 100 different observers were responsible for the Greenwich data. In 1850 the instrument used was changed from the 5 inch transit to the 8 inch Airy transit circle. Also the means of recording the transit has varied. These changes complicate the interpretation.

So the mystery remains. Eddy and

Boornazian are convinced that the sun is shrinking and claim that the fact that the 9 April 1567 solar eclipse was annular and not total indicates that the sun was bigger in the past. The situation is complicated by cyclic variations. Secchi and Rosa suggested in 1872 that the solar diameter varied with the 11 year sunspot cycle. Cimino (1945) and Giannuzzi (1955) found a 22 year period.

Looking to the future the SCLERA (Santa Catalina Laboratory for Experimental Relativity by Astrometry) f/100 photoelectric telescope could be used to measure the diameter to an accuracy of 0.01 arc s, a twenty-fold improvement in sensitivity, so the problem of size variation should be placed on a much sounder footing. The weather and sky transparency are better in Tucson, Arizona than in Greenwich or Washington and this should help too.

Structure of bacteriophage Pf1

from R.A. Crowther

A NEW structural model for bacteriophage Pf1, differing in potentially important ways from that previously accepted, has just been proposed by Makowski, Caspar and Marvin (*J. molec. Biol.* **138**, 149; 1980).

Bacteriophage Pf1 is representative of a class of filamentous virus particles which infect gram-negative bacteria. The particles, which are about $1 \mu\text{m}$ long and 60 Å in diameter, contain a closed circular single-stranded DNA molecule of about 7,000 bases. The DNA is coated by many copies of a small protein of known sequence with a predominantly α -helical conformation. There is about 1 base of DNA per coat protein in Pf1, though the ratio is different in other phage, suggesting that interactions between DNA and coat may not be very specific. The ability to package single-stranded DNA molecules of arbitrary size makes filamentous phage, particularly M13, attractive as vectors for DNA cloning and sequencing.

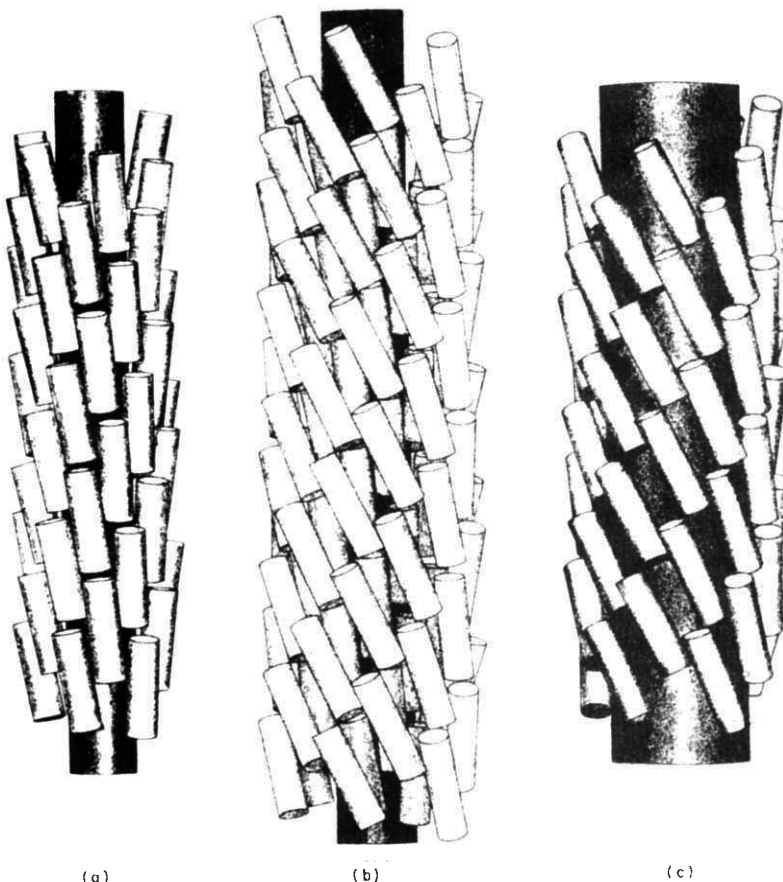
An elongated structure built from a repeated unit would be expected to have helical symmetry and the X-ray diffraction patterns obtained from Pf1 confirm this. The detailed interpretation of the X-ray pattern has however been more controversial. Marvin and Wachtel (*Nature* **253**, 19; 1975) proposed a model for Pf1 assuming a helical repeat containing 22 coat protein molecules in 5 turns of a 15 Å pitch helix. The model represented the coat subunit as a single α -helix coiled into a segment of a left handed concho-spiral. This interpretation was questioned by Makowski and Caspar (In *The single stranded DNA phages* eds. Denhardt, Dressler & Ray, p.627; 1978),

who presented evidence favouring a subunit repeat of 27 units in 5 turns of a 15 Å pitch helix. The new model proposed jointly by the two groups is based on the latter assignment of helical symmetry, although the local structure is not much

affected.

The X-ray diffraction pattern provides only part of the information needed to compute a map of the structure. Although the intensity of the various parts of the pattern is recorded the relative phase

Fig: The arrangement of α -helical segments in the virus particle. Two axial repeats of the inner layer of rod segments are shown in (a), two repeats of the outer layer in (c) and 2.5 repeats of both layers in (b). The α -helical segments in the virus protein subunit are represented as cylinders around the central core, occupied by DNA in the virus particle.



R.A. Crowther is in the MRC Laboratory of Molecular Biology, Cambridge.

information is lost and must be regenerated by indirect means, such as introducing heavy atoms into the structure. This has not yet been possible for Pf1, so a more circuitous approach has been adopted by Makowski *et al.*, involving cycles of model building and iterative phase refinement. The stereochemical constraint of α -helical structure and the geometrical 'box' constraint of a particle of limited radius, within which all the scattering material must be confined, combine to produce what the authors claim is a unique structural solution consistent with the X-ray intensities to spacings of about 7 Å.

The picture that emerges of the coat protein (see figure) is of a segmented structure. One set of α -helical segments lies almost parallel to the particle axis at a radius of about 15 Å, while the other set of α -helical segments lies at about 25 Å radius and is tilted by about 25° to the particle axis. The resulting tightly packed and intricately interlocked structure conforms with accepted models for packing of

α -helices and provides a stable 20 Å thick protein coat around the viral DNA. Unfortunately the model is not yet sufficiently detailed for the connectivity of the segments to be established. An inner segment might connect to an outer segment lying further along the virus giving an elongated though kinked subunit, reminiscent of the single α -helix of Marvin & Wachtel. More intriguingly the connectivity might be such that an inner segment joins to the outer segment overlying it at larger radius, giving a hair-pin fold to the molecule. The latter would produce a very compact coat protein, resembling the double α -helical hair-pin proposed as the primitive ancestor of the present day tobacco mosaic virus coat protein (McLachlan *et al. J. molec. Biol.* 136, 203; 1980). More detailed studies are needed to choose between these possibilities but the outcome will clearly provide an important constraint on the molecular mechanisms proposed for viral morphogenesis. □

they were generally located. The erect forms were associated with habitats in which catastrophes were common, such as disturbed soils, and devoted a greater proportion of their energy to reproduction. The erect types can therefore be regarded as r-selected with respect to the prostrate types.

A subsequent analysis of populations of the broad-leaved plantain, *Plantago major*, has demonstrated some similarities to and some differences from the meadow grass (*New Phytol.* 85, 289; 1980). Populations were examined from regularly mowed lawns and from waste ground beside roads and canals. Again, there was a differentiation into populations with flattened, prostrate rosettes of leaves (found on lawns) and those with erect, tall shoots and leaves (found on roadsides). Naturally, when exposed to a régime of regular clipping at a height of 2 cm above ground level, the erect form suffered far more severe damage than the prostrate form. In this respect the plantains resembled the meadow grass. A consideration of Warwick and Briggs' data concerning the accumulation of vegetative and reproductive dry weight over the 14 weeks of the experiment, reveals some interesting contrasts to the meadow grass observations. Under control (unclipped) conditions, the reproductive structures (flower and fruiting spikes plus their scapes) of the lawn plantains account for between 62% and 65% of the total plant dry weight. The roadside populations, however, have a generally lower and more variable reproductive component, between 28% and 47% (based on population means). Thus, under control conditions, the prostrate form exhibits a greater degree of energy diversion to reproduction than the erect population, and this character is normally an indication of r-selection. Under clipped conditions, as one might reasonably predict, this difference is further exaggerated, for the shorter, less erect spikes of the lawn type are far more successful. After 14 weeks growth under a weekly mowing regime, the lawn population had a 52% to 54% investment in reproductive structures and the roadside plants were reduced to 13% to 17%.

Such clear indications of ecotypic variations have not, however, been found in all the lawn weeds examined by Warwick and Briggs. In the daisy, *Bellis perennis* (*New Phytol.* 85, 289; 1980), there was a great deal of variation within populations from grasslands of differing mowing and grazing histories. Transplantation into a regularly cut lawn did not necessarily diminish the reproductive vigour of some individuals from grazed habitats, but, in the case of the daisy, the importance of vegetative reproduction in the overall success of the plant is much greater than for either the plantain or the meadow grass. One cannot measure the full potential of a weed simply by its commitment to sexual reproduction. □

Plant opportunists

from Peter D. Moore

ADAPTABILITY is a valuable asset for an opportunist. The maxim applies as well to the struggling weed as to the business entrepreneur, as an increasing number of studies of the genetics and population biology of plant pest species is showing. Much of the interest in this area of botany stemmed from Bradshaw's work on variation within grasses such as *Argrostis tenuis* (see for example, *New Phytol.* 59, 92; 1960) in response to intense selective pressures operating over limited spatial distances. In particular, it was the study of heavy metal pollution in areas disturbed by mining and dumping which led him and his students to an awareness of the genetical mosaic found in the plant cover as a response to such soil stresses (Jain & Bradshaw *Heredity* 21, 407; 1966). In some cases it was possible to pinpoint the precise physiological adaptation involved in the provision of selective advantages, as in the case of Snaydon and Bradshaw's work (*New Phytol.* 60, 219; 1961) on ecotypic variation in the grass *Festuca ovina* on alkaline and acid soils. Here, membrane selectivity (between sodium and potassium) was shown to be closely correlated with the environmental pH.

Complex variations and patterns in the micro-environment are frequently associated with human activity, as in the case of mine spoil, and consequently one

might reasonably expect to find genetic diversification and intense local selection within those plant species which are especially dependent on man's activity, namely the weeds. This has indeed proved to be the case, for example, the work of Gadgil and Solbrig (*Am. Nat.* 106, 14; 1972) on dandelions in North America, showed that these plants form a patchwork of genetically uniform populations, each of which has characters of growth, reproductive rate and seed size which could be regarded as adaptive responses to their varying local environment, such as degree of disturbance and frequency of mowing. (see *Nature* 252, 191; 1974, 269, 375; 1977).

But dandelions are somewhat peculiar in that they are apomictic and also can regenerate very effectively by vegetative means. Both of these characteristics lead to the conservation of a uniform genetic make-up within populations. Much interest has therefore been generated in the degree of variation and adaptation in other common weed species. An interesting series of papers concerning the genecology of a number of weeds has been published by Warwick and Briggs, who have been particularly interested in lawn plants. In the annual meadow grass, *Poa annua*, they found that some plants were prostrate and others erect, and that this growth habit was genetically determined (*New Phytol.* 81, 711; 1978). The prostrate types were better able to withstand defoliation and were well adapted to lawn conditions, which is where

Peter D. Moore is in the Department of Plant Sciences, University of London King's College.

New developments in iron-sulphur proteins

from R. Cammack

A NOVEL kind of iron-sulphur cluster has been found in a ferredoxin from the nitrogen-fixing bacterium *Azotobacter vinelandii*. The cluster is unusual in that it contains a ring of three iron atoms linked by labile sulphide atoms. The iron-sulphur proteins are a class of proteins that contain clusters of iron atoms bound to cysteine and sulphide sulphur. They are an important group of biological electron transfer agents. For example, more than a dozen different types of iron-sulphur clusters have been detected in mitochondria where they are concerned with energy metabolism. Two years ago, the possibility that iron-sulphur clusters can have other functions was raised when F. J. Ruzicka and H. Beinert (*J. Biol. Chem.* **253**, 2514; 1978) found that aconitase is an iron-sulphur protein. This enzyme of the Krebs tricarboxylic acid cycle of mitochondria has no known electron transfer function. It now seems likely that the iron-sulphur cluster in this enzyme is unusual in another respect, in that it may be a new type of three-iron cluster, similar to those recently proposed in certain bacterial proteins.

In the early 1970's it became established, by a combination of X-ray crystallography, spectroscopy and synthesis of chemical analogues, that all the iron-sulphur proteins contained either 1-iron, (Rd), 2-iron [2Fe-2S] or 4-iron [4Fe-4S] clusters (see figure). The 1-iron centres are found only in the rubredoxins of certain bacteria but the [2Fe-2S] and [4Fe-4S] clusters, in which iron is attached to sulphide sulphur and cysteine sulphur from the protein, has been found in many electron-transferring systems and enzymes. The iron-sulphur proteins are often described by the low-temperature electron-paramagnetic-resonance (EPR) spectra, since this is a good way of detecting them in biological systems. The [2Fe-2S] clusters and some of the [4Fe-4S] clusters give EPR signals in the reduced state at $g=1.94$. Other [4Fe-4S] clusters

give EPR signals around $g=2.01$. However recent results suggest that a novel type of cluster may also give a $g=2.01$ signal.

The centre of attention is a ferredoxin from *Azotobacter vinelandii*. This protein contains two iron-sulphur clusters with widely different redox potentials, +320mV and -424mV. Both give EPR signals at $g=2.01$. The first cluster seems to be a conventional [4Fe-4S] type. The second one, however, seems to be different. Chemical analysis (which is, admittedly, notoriously difficult with these proteins) indicates that it contains fewer than four iron atoms. Preliminary X-ray crystallography of the ferredoxin at 4 Å resolution by C. D. Stout of the University of Pittsburgh showed that this cluster was smaller than the other and last year it was proposed (Stout *Nature* **279**, 83; 1979) that it might be a two-iron cluster. A clearer picture of the cluster has now emerged from an electron density map at 2.5 Å resolution (Stout, Ghosh, Patabni & Robbins *J. Biol. Chem.* **255**; 1979; 1980).

A model (see figure) has now been fitted to the X-ray data and can be considered as a two-iron cluster which has opened at one side to introduce a third iron atom (numbered 3) and sulphide atom. Unlike all other iron atoms in iron-sulphur clusters, atom 3 does not have tetrahedral symmetry but appears to be almost square planar. Also, the ligands from the protein could not all be cysteine sulphurs as there are insufficient residues of this amino acid available.

The results of the X-ray analysis are still preliminary. Refinement of the full structure of the protein is not expected before the end of this year. However support for the existence of a three-iron cluster is provided by a totally different technique, ^{57}Fe Mössbauer spectroscopy. A paper from groups in Wisconsin and Minnesota (Emptage, Kent, Huynh, Rawlings, Orme-Johnson & Münck *J. Biol. Chem.* **255**, 17973; 1980) looks at the iron atoms in *A. vinelandii* ferredoxin. After subtracting the spectrum of the [4Fe-4S] cluster, the second cluster appears to contain two types of iron atom, in the ratio 2:1. As in other iron-sulphur clusters, the

iron atoms are antiferromagnetically coupled together. The observation that the cluster gives the EPR signal at $g=2.01$ in the oxidized form, but is non-magnetic in the reduced state, can readily be explained by a three-iron cluster. (This was a difficulty with the previously proposed two-iron cluster). The valence states of the iron atoms would correspond to 3Fe^{3+} in the oxidized state, and 2Fe^{3+} and Fe^{2+} in the reduced state, giving net charges for the cluster of +3 and +2, respectively.

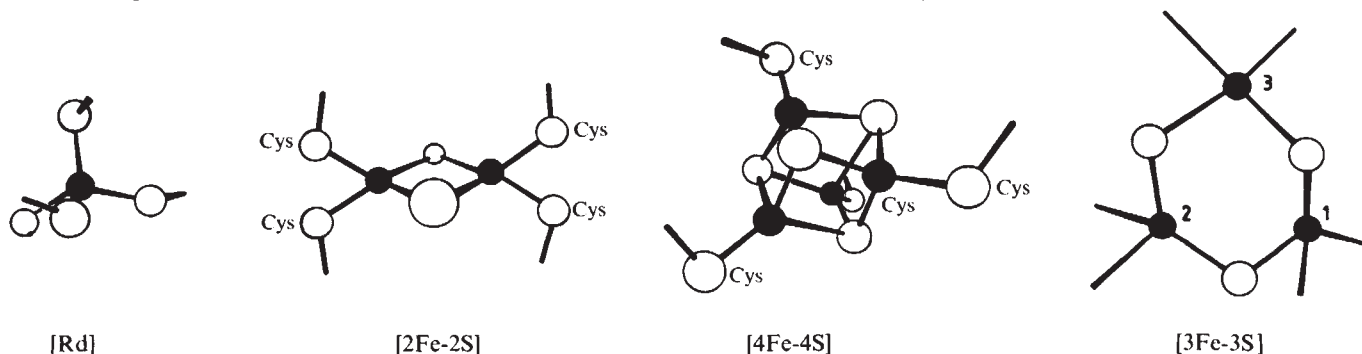
If the existence of three-iron clusters is confirmed, there are already a number of proteins known that may also contain them. All of these given the EPR signal at $g=2.01$. Mössbauer spectra of ferredoxin II from *Desulfovibrio gigas* indicate that it contains a [3Fe-3S] cluster on each polypeptide chain. Since the protein is a tetramer it appears to contain four such clusters (Interestingly ferredoxin I from the same organism contains [4Fe-4S] clusters in a trimeric unit of exactly the same polypeptide). Other, more complex proteins, may also contain the cluster. Emptage *et al.* report that they have observed similar Mössbauer spectra from the enzyme glutamate synthase of *A. vinelandii* and from aconitase of beef heart mitochondria.

The presence of this iron-sulphur cluster in aconitase is intriguing. The cluster does not appear to participate in the enzyme reaction directly, but the enzyme is inactive unless the cluster is first reduced. It has therefore been suggested that the cluster somehow regulates the activity of the enzyme. If this idea becomes accepted, there are other examples known. Glutamine amidoribosyl transferase is an enzyme concerned with nucleic acid synthesis. The enzyme, both from human tissue and from *Bacillus subtilis* has been found to contain an iron-sulphur cluster (possibly of the [4Fe-4S] type). Again this has no obvious role in catalysis, but the enzyme is only active when the cluster is reduced.

It seems, as more types of iron-sulphur proteins continue to be discovered, that we have much to learn about the versatility of iron-sulphur clusters. □

R. Cammack is a Lecturer in the Department of Plant Sciences, University of London King's College.

Atomic arrangements in the one-iron (rubredoxin, Rd) two-iron and four-iron clusters, and the proposed three-iron cluster.





100 years ago

NEW SCHEME FOR DIRECTING BALLOONS

M. Gabriel Yon, one of the directors of the great Giffard captive balloon, and Mr. E.A. Pearce of Bristol, have each published a pamphlet on the direction of aërostats. The balloon of each inventor is to be elongated according to the principles of the experiments tried by Giffard in 1852 and by Dupuy de Lôme in 1871. The propeller is to be moved by a gas-engine in the Pearce balloon, and by a steam-engine in the Yon balloon. M. Yon proposes to use the gas of the balloon as fuel, but only in proportion to loss of weight produced either by uncondensed steam or by consumption of petroleum.

Nothing can be said to be really impracticable in the Pearce scheme, although Mr. Pearce lacks the aeronautical training which distinguishes M. Yon,

an aeronaut who ascended with M. Giffard in 1854, and has witnessed all his experiments. The only essential difference between M. Giffard's scheme and the new system is the place given to the fan, which M. Giffard attaches to the car. Practice will only decide whether the alteration projected is an improvement or otherwise. The reason alleged for the change is the bringing of the fan nearer to the centre of resistance. But it obliges the aeronaut to give to his fans a very small diameter, which requires an immense number of rotations in a second, and consequently represents a loss of power.

The calculations appear to have been made with care by M. Yon and Mr. Pearce. A trial would be greatly desirable, although it is impossible to suppose that the aerial carriage of Mr. Pearce or the directing balloon of M. Yon can possibly bring aeronauts to the North Pole for their inaugural trip, they may be instrumental in eliciting useful facts. We may add to the peculiarities of M. Yon's scheme that he uses a small globe inclosed in the lower part of the aërostat called a compensation sphere, and connected by a pipe with a ventilator, for keeping intact the form of his aerial machine. Mr. Pearce does not appear to be convinced of the urgent necessity of abstaining in any aerial construction from every complication which can be avoided at any cost, and he suggests the adoption of some accessory organs which, although designed to help

aëronauts, would tax too much the lifting power or the attention of the aerial sailor. Mr. Pearce supposes that he will be able to navigate the air with an excess of weight, and does not pay attention to the intensity of motive power required to counteract gravitation even in a partial manner. He should certainly take advantage of the pamphlet written by his French competitor, who deals mostly with facts belonging to the public, and on which nobody can, in the present state of science, raise any claim as being his own property.

Both these pamphlets are greatly in advance of similar productions, and are creditable to their writers. Mr. Pearce's pamphlet has been only published for private publication. M. Yon's is printed with a number of plates representing many details; but a directing balloon is so complex a matter that this part of the publication can hardly be said to be complete.

Having been the builder of M. Dupuy de Lôme's balloon and one of his crew, M. Yon may be said to have witnessed all the great aeronautical constructions of the age. Next to M. Henry Giffard, of whom he claims to be the pupil, he is the most completely qualified aeronaut to work out the solution of the great problem to which a recent success in photography has given unexpectedly in some respects a practical result.

From *Nature* 22, 29 July, 303, 1880.

The mechanism of two-nucleon transfer reactions

from P.E. Hodgson

SOME recent calculations made by Da Hsuan Feng *et al.* (*Phys. Rev. Lett.* 44, 1037; 1980) greatly clarify the interpretation of two-nucleon transfer reactions. These reactions, particularly the (p,t) and the inverse (t,p) reactions, are extensively used to study nuclear structure but the interpretation of the experimental results has been hindered by lack of knowledge of the reaction mechanism. The distorted wave calculations of the reaction cross-sections tend to give the shapes of the angular distributions rather well, but the absolute magnitudes are very often much smaller than the experimental values. The situation compares unfavourably with that for the corresponding one-nucleon transfer reactions, which can usually be accounted for both in angular distribution and in absolute magnitude using the distorted wave theory.

Attempts to improve the calculations of the cross-section of the two-nucleon transfer reactions fall into two categories, those that concentrate on the nuclear reaction and those that concentrate on the nuclear structure. It has been found that the inclusion of additional reaction mechanisms — for example a two-stage process through an intermediate nucleus — gives additional contributions to the cross-section that brings it into closer accord with the experimental values. Another line of research is to include more detailed wavefunctions for the initial and final nuclear states, and these also tend to increase the calculated cross-section. There still remains, however, much uncertainty about the reaction mechanisms that have to be included, and the importance of higher-

order nuclear configurations.

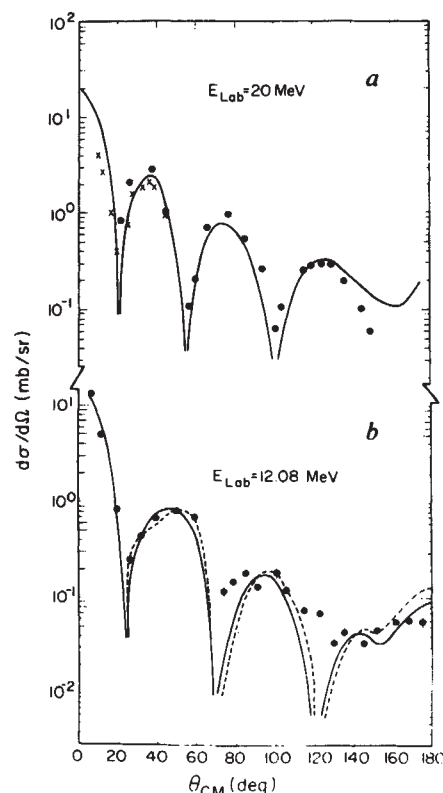
Da Hsuan Feng *et al.* use realistic triton and nuclear wave functions and succeed in fitting the experimental cross-sections both in magnitude and in angular distribution. The reaction was assumed to take place by the direct pickup of the two nucleons from the target nucleus by the incident proton to give the outgoing triton. The wavefunction of the proton and the triton are generated by the appropriate optical potentials, and the reaction matrix element is evaluated without making the assumption that the interaction potential has zero range, an assumption that is known to give good results for one-nucleon transfer reactions but not for two-nucleon transfer reactions.

The improved form of the triton wave function is an important feature of the new calculations. Most of the previous calculations included only the predominant spatially symmetric S state. But now the mixed-symmetry S' and D states are included as well. This alters the selection rules and allows transitions to unnatural parity states to take place by a one-step process. The overlap integral between the wavefunctions of the initial and final nuclei satisfies a Schrödinger equation for two interacting particles in an external potential, and this was solved by an extended-basis shell model method. Overall, these new calculations are the first that use realistic transfer potentials, triton wavefunction and nuclear overlap functions and are thus the first detailed test of the validity of the distorted wave method as applied to two-nucleon transfer reactions.

To evaluate the success of this formulation, it was applied to the $^{18}\text{O}(p,t)^{16}\text{O}$ reaction at 20 MeV., the $^{48}\text{Ca}(t,p)^{50}\text{Ca}$ reaction at 12.08 MeV. and

the $^{90}\text{Zr}(t,p)^{92}\text{Zr}$ reaction at 11.89 and 20 MeV., and the results for the first two of these reactions are compared with the experimental data in the figure. In both cases, and also for the third reaction, the

Figure: (a) Differential cross-section for the $^{18}\text{O}(p,t)^{16}\text{O}$ reaction compared with distorted wave calculations. (b) A similar comparison for the $^{48}\text{Ca}(t,p)^{50}\text{Ca}$ reaction. The dashed curve shows the result of using a different way of calculating the wavefunctions of the transferred nucleons.



P.E. Hodgson is in the Department of Nuclear Physics, University of Oxford.

agreement is good both for the absolute magnitude of the cross-section and also for its angular variation. Since the optical potentials are obtained from the analysis of elastic scattering cross-sections, and the parameters of the nuclear structure part of the calculation are fixed in advance, there are no adjustable parameters in the calculations. The excellent fit to the data for different reactions at two energies for different nuclei shows that the essential features of the reaction have been included in the calculation. In particular, the results provide no evidence in favour of the suggestion that two-stage processes

contribute appreciably to the cross-section.

Further work remains to be done to test these calculations against a wider range of data, and also to improve the method still further. In particular, the validity of the distorted wave method needs to be examined in more detail, and account needs to be taken of the possibility of exchanges between the ingoing proton and the target nucleus. However even in its present form the calculation is a significant advance on previous work, and should provide a powerful tool for nuclear structure studies. □

Evolution and gaps in the fossil record

from Mark Ridley

CHARLES Darwin's theory of evolution differed from most of its alternatives in postulating that new parts evolved in many tiny stages from previously existing parts. Unfortunately, the intermediate stages hardly ever seemed to exist in the fossil record (Huxley's later trumpeting about *Archaeopteryx* notwithstanding). Darwin himself followed Lyell in blaming the fossil record — it was incomplete, unhistorical and misleading, so it did not count against this theory. If taken to an extreme the two ideas (that the fossil record is incomplete and that evolution proceeds at a fairly constant rate) can form an irrefutable system in which gradual temporal change in fossils is interpreted literally, but sudden jumps between species are attributed to gaps in the record. All the evidence is then consistent with a constant tempo of evolution.

A few years ago Eldredge (*Evolution* 25, 156; 1971) and then Eldredge and Gould (*Models in Paleobiology* ed. J. W. Schopf; Freeman, San Francisco, 1972; *Paleobiology* 3, 115; 1977), following on suggestions by Mayr, Simpson and others, argued that the gaps in the fossil record might be more real than apparent. They put forward a version of the theory of allopatric speciation that would automatically generate gaps in the fossil record. According to their theory: (1) the intermediates between species existed in small populations, and small populations are less likely to leave a record than large ones (2) evolution is rapid in the small isolated population, and (3) the intermediates did not exist in the same place as (and so are unlikely to be preserved with) their ancestor. Evolution, therefore, is not gradual and of constant tempo. There are short periods of rapid change at speciation, separated by longer periods in which there is no change. Eldredge and Gould christened this the 'punctuated

equilibrium' model of evolution, and its alternative 'phyletic gradualism'.

Eldredge and Gould's forceful essay has inspired two controversies. One is over whether the fossil record does, in fact, show one pattern or the other, and the second concerns the validity of Eldredge and Gould's explanation of punctuated equilibria. Even if it is established that evolution is punctuated, Eldredge and Gould's theory of speciation is only one of a variety of possible explanations.

Biometrical studies, in which the range of variation is described for each interval within a continuous sedimentary unit, are essential for a test between phyletic gradualism and punctuated equilibria. Incompleteness of the record and typological taxonomy (in which each specimen is named by its similarity to a type specimen) will otherwise be confounding factors. A study of geographical variation is also desirable. If the samples contain specimens from the ancestral species pooled with increasing (as time passes) numbers from the allopatrically derived species, then gradual evolution can appear as an artefact even if the actual evolution was punctuated. Such pooling is less likely to occur if geographic variation is studied separately.

Gingerich has published several biometrical studies of mammal evolution, though his studies lack information about geographical variation (*Am. J. Sci.* 276, 1; 1976; *Pap. Paleont. Mus. Paleont. Univ. Mich.* 15, 1; 1976; *J. Paleont.* 53, 153; 1979; Gingerich & Symons *Contr. Mus. Paleont. Univ. Mich.* 24, 245; 1977). His most abundant data are for the condylarth *Hyopsodus* in the early Eocene (*Am. J. Sci.* 276, 1; 1976), which looks as if it evolved gradually. Gould and Eldredge (*Paleobiology* 3, 115; 1977) said that the rate of evolution in *Hyopsodus* was too slow to be important. However, the rate of change within a *Hyopsodus* species is similar to that at speciation. This is manifest phyletic gradualism even if only on small scale.

More recently, West (*Paleobiology* 5, 252; 1979) followed the descendants of Gingerich's *Hyopsodus* through a middle Eocene formation of at least one million years, and found no evidence of evolutionary change. So even if *Hyopsodus* did evolve gradually in Gingerich's study site, they showed prolonged stasis soon afterwards. Gingerich's other papers document phyletic gradualism in other condylarths and in primates, and there is now a steady trickle of papers (see recent issues of *Paleobiology*) variously substantiating either gradual or punctuated change.

Eldredge and Gould's microevolutionary theory has also been criticized. The first problem is whether speciation occurs in small populations. Anderson and Evensen (*Syst. Zool.* 27, 421; 1978) have compared the sizes of the geographic ranges of recently diverged pairs of species of North American terrestrial vertebrates and found that "more than half of the recently divergent species pairs have the range of the smaller species greater than 30 percent of that of the larger species". Although uncertainty exists about just how recently the pairs of species diverged and about the relation between range and population sizes, Anderson and Evensen's data does not support Eldredge and Gould's claim that speciating populations are very small. It can also be asked whether evolution is rapid in small populations. Smaller populations have lower total rates of mutation which can cause lower rates of evolution (e.g. Maynard Smith *Am. Nat.* 110, 331; 1976).

The second problem is to reconcile punctuated evolution with natural selection and genetics. The rate of evolution might either be controlled by the extent of genetical variation, or by the intensity of selection. On the latter possibility, the pattern of evolution is directly controlled by rates of change of the environment. The *Gryphaea* studied by Hallam (*Paleobiology* 4, 16; 1978), for example, speciate rapidly when their ranges are fragmented by a fall in sea level. When the sea rises *Gryphaea* gradually evolve increased size. The other hypothesis is that rapid evolution results from rare, large mutations; chromosomal changes are possible examples. Chromosomal differences between species are well-known in a variety of taxa, and several authors have broached chromosomal mechanisms for rapid speciation in small populations (reviewed by M.J.D. White *Modes of Speciation* W.H. Freeman, San Francisco; 1978), which would cause a 'punctuated' pattern of evolution. One suggested mechanism is a change in a chromosome (such as a translocation or an inversion) within one local population. Heterozygotes for the mutation would probably be selected against, so that if members of this population interbred with the ancestral species there would be intense selection for reproductive isolation. However, even given a correlation between chromosome

differences and speciation, it is not obvious which is cause and which effect, and nor can we be sure that chromosomal speciation would necessarily produce punctuated evolution. In his latest essay on the subject, Gould (*Paleobiology* 6, 119; 1980) argues that large mutations are the main cause of punctuated evolution.

A third and interesting possibility is that gradual environmental change could cause punctuated evolution. In their original paper, Eldredge and Gould mooted that a species' gene pool is so intricately co-adapted that change is only possible by a thorough revolution. By co-adapted it is meant that a change in one part of the system will not be favoured without simultaneous changes in other parts. Thus when evolution occurs it will be by a sudden leap, with changes in many parts; natural selection does not favour piecemeal tinkering.

Ironically, an objection to any model in which punctuated evolution occurs with gradual environmental change comes from the very facts that led to the formation of the theory to begin with: geographical variation. Stepped clines (discontinuous variation in space) are known that do not

coincide with obvious environmental discontinuities, such as the enigmatic spotting pattern of the butterfly *Maniola jurtina*, and these have been interpreted as quantum jumps between different co-adapted genetic systems (E.B. Ford *Ecological Genetics* Chapman & Hall, London; 4th edn, 1975, J.A. Endler *Geographic variation, speciation, and clines* Princeton University Press, 1977). However, continuous variation in space is commonly observed in many species and is difficult to reconcile with the theory that says that natural selection causes abrupt changes between different co-adapted states. Earlier this century gradual geographical variation was important in confuting the de Vriesian 'mutationist' theories of evolution. Gradual geographic variation probably evolves much too rapidly to be represented in the fossil record as gradual evolution, so the existence of gradual geographic variation is perfectly compatible with punctuated evolution over geological time. Gradual geographic variation, however, does not fit in well with the 'co-adaptation' theory of how natural selection brings this punctuated evolution about. □

west of the 1946 Nankaido earthquake were reported by Mogi (Tokyo University). He also showed how a recent model by Shimazaki and Nakata, in which rupture occurs at a constant ultimate stress (as opposed to a model of constant stress drop during an earthquake), can be used to explain the observation that the time interval between large earthquakes in western Japan is proportional to the stress drop in the preceding large earthquake. Takagi (Tohoku University, Japan), in a detailed study of activity before and after the 1978 magnitude 7.4 Miyagi-oki earthquake, showed a migration of foreshocks towards the boundary between the oceanic slab and overriding plate, with aftershocks clustering along the boundary. Seismicity patterns in the Mexico-Central America segment of the circum-Pacific belt were described by McNally (California Institute of Technology), Ohtake *et al.* (National Research Center for Disaster Prevention, Japan) and Singh *et al.* (Instituto Geofisica, Mexico), who reviewed data which led to the successful prediction of the 1978 Oaxaca earthquake and showed repeat times of 30-40 years for this region. For at least 50 years following the 1906 San Francisco earthquake the level of seismicity in northern California was considerably lower than it had been in the preceding 50 years. Ellsworth *et al.* (US Geological Survey) suggested that a pair of magnitude 5.5 earthquakes in the San Francisco area in August 1979 and January 1980 show the activity to be returning to a more normal level. Pairs of earthquakes with similar temporal clustering have occurred a number of times in the area since 1836.

In continental areas, where the repeat times for large earthquakes are longer than for oceanic subduction zones and are usually beyond the range of instrumental or historical catalogues, geological data are being used to extend the record of occurrence of major earthquakes. Sieh (California Institute of Technology), Wallace (US Geological Survey) and Schwartz *et al.* (Woodward-Clyde consultants, San Francisco) showed how detailed mapping of displacement on major fault zones in the western US can be used to date past earthquakes to determine recurrence intervals and maximum magnitudes of prehistoric events. They showed a general increase in the recurrence interval from west to east: from tens to hundreds of years for large earthquakes along the San Andreas system to hundreds to thousands of years in Nevada and Utah.

Whereas most methods for long-term prediction are based on regularities and changes in the seismic cycle itself as revealed

Earthquake prediction

from David W. Simpson

In the early 1970s, principally because of observations of premonitory changes in velocity ratio and groundwater geochemistry in the USSR and crustal deformation studies in Japan, there was considerable optimism that earthquake prediction would soon become a reality. The intervening years have seen a greatly expanded program in earthquake prediction in the United States, but negative and conflicting results have dampened some of the initial enthusiasm, if not led to outright pessimism on the part of many US seismologists regarding the practical reality and imminence of reliable methods for predicting earthquakes. At a recent meeting* on a wide variety of topics related to earthquake prediction much of the original enthusiasm appeared to be re-emerging as it became clear that at least some earthquakes can be, and in fact have been, successfully predicted. It was also clear, however, that no universal panacea can be expected. Premonitory effects appear to vary widely with tectonic environment and earthquake magnitude. The premonitory signatures of large earthquakes in thrust regions of Soviet

Central Asia, China and Japan appear to be clearer and to extend over a wider area than any such effects observed so far in strike-slip environments such as the San Andreas Fault of California.

On a time scale of tens of years, gaps in the occurrence of large earthquakes along major plate boundaries have proved to be one of the most successful methods for predicting the sites of future great shocks. Nishenko, McCann and Sykes (Lamont-Doherty Geological Observatory, Columbia) presented a map of the current 'seismic potential' of the circum-Pacific belt, in which they refined the idea of seismic 'gaps' by ascribing a high seismic potential to a gap only if historical or tectonic data indicate that it is a region capable of producing a large shock. Sykes *et al.* (Lamont-Doherty) used historical reports to extend the record of great earthquakes in the Alaska-Aleutian arc back to 1784 and showed repeat times of 50 to 100 years for great earthquakes in some regions. Large segments of this part of the plate boundary seem to rupture in temporally clustered sequences of large earthquakes (e.g., 1898-1907 and 1938-1965), separated by long periods of relative quiescence. Variations in seismicity in western Japan, and especially a south to north migration in the area to the

David W. Simpson is in the Lamont-Doherty Geological Observatory of Columbia University, New York.

* The Third Maurice Ewing Symposium was held at Mohonk Mountain House, New Paltz, New York. Eighty-eight scientists from eleven countries attended under the sponsorship of the Lamont-Doherty Geological Observatory of Columbia University, with Lynn R. Sykes as Chairman of the Organizing Committee. Support was provided by the G. Unger Vetlesen Foundation, the National Science Foundation and the U.S. Geological Survey. The Proceedings will be published in 1981 by the American Geophysical Union.

Insect diapause — stability and change

from A.D. Lees

GEOGRAPHICAL strains of insects often differ substantially in their diapause characteristics. The critical photoperiod required for diapause induction, the interactions between day length and temperature and the intensity (duration) of dormancy are particularly variable. When the area of distribution extends continuously over a wide range of latitudes these characters form a north-south cline with critical photoperiod and intensity diminishing towards lower latitudes and diapause sometimes fading out entirely in the sub-tropics. Following the classical work of Danilevsky (*Ent. Obozr.* 36, 6; 1957), this has been accepted as evidence that geographical strains are closely adapted in their phenology (date of entry into diapause, voltinism etc.) to their own part of the geographical range of the species. Since photoperiod and temperature, as the main agents controlling diapause, vary systematically with latitude, the insect would receive a false indication of season in other parts of the range. This is expected to limit spread along a north-south axis and might have severe initial effects on introduced species, although some later readjustment by natural selection might well be looked for. In this connection, crosses between different strains have shown that quantitative differences in diapause-related characters

such as critical photoperiod and diapause intensity depend on polygenic inheritance. Nevertheless, as emphasised by Tauber and Tauber (*Evolution of Insect Migration and Diapause* p.53-71, Springer-Verlag, 1978), these features can sometimes be modified quite rapidly by directional selection in the laboratory.

Introduced pests that fail to gain a foothold usually escape notice. For this reason the diapause status of the more successful invaders is especially worthy of scrutiny. In this issue of *Nature* (p.489) Goldson and Emberson show that the Argentine grass stem weevil *Hyperodes bonariensis*, introduced into New Zealand more than 50 years ago, has a remarkably stable photoperiodically controlled adult diapause, populations collected in areas extending over 8° of latitude having much the same critical photoperiod. It seems that the beetle might have achieved multivoltinism (and even greater destructiveness?) if diapause had been eliminated by selection. The absence of diapause in most indigenous New Zealand insects suggests that this condition is not required for winter survival.

In contrast, the photoperiodically controlled winter diapause of the Colorado potato beetle is well suited to its new environment in continental Europe. Introduced into France in 1922, *Leptinotarsa* has since spread eastwards as far as the USSR, occupying only slightly wider latitudinal limits than in N. America. It seems likely that the diapause

control mechanisms and the voltinism have again remained relatively stable (de Wilde, *Soc. exp. Biol. Symp.* 23, 263; 1969). The pink bollworm *Pectinophora gossypiella*, originating in India, is a pest which has been transported by commerce to virtually every cotton growing area in the world, mainly during the present century. The geographical strains tested by Ankersmit and Adkisson (*J. Insect Physiol.* 13, 553; 1967) were found to have very similar critical photoperiods for diapause induction even though the populations came from widely separated latitudes (for example, El Paso 32°N and Colombia 3°N). However, many fewer insects from lower latitudes entered diapause at all, even in short days. In this species adaptive changes are already far advanced. In the cornborer *Pyrausta nubilalis* the evidence suggests that changes in the diapause status are progressing rapidly. Introduced into N. America from Europe before 1920, there has been a strong tendency for the univoltinism of populations in the north to give way to a bi- or multivoltine life cycle in the warmer states of Kansas and Missouri. According to the analysis of Beck and Apple (*J. econ. Ent.* 54, 550; 1961), these changes involve both photoperiod and temperature control of the inductive daylength as well as more indirect effects of temperature on the rate of larval development. From these few examples it is clear that the adaptive changes shown by different insects are liable to be varied and complex. □

A.D. Lees is Professor of Insect Physiology, Imperial College, London.

by seismicity data, many intermediate- and short-term predictions rely strongly on indirect indicators of the earthquake preparation process. Aki (Massachusetts Institute of Technology) presented a method by which information from various independent precursors can be combined to improve estimates of the probability of the occurrence of a large earthquake. Thatcher (US Geological Survey) showed how geodetic measurements have been useful, especially in Japan and California, in identifying areas of increased stress as the possible sites for future earthquakes. The importance of inhomogeneities in the stress distribution on fault zones and the role of asperities in controlling the release of stress were discussed by Kanamori and Lay (California Institute of Technology) and Wyss (Institute für Geophysik, Zurich). A model of time dependent failure due to stress corrosion, in which the growth of cracks is controlled by a 'stress intensity factor' and the presence of asperities, was presented by Scholz *et al.* (Lamont-Doherty).

In short-term predictions, water-related effects are especially common. A number of papers described the observation of changes

in radon content in groundwater and deep wells. Fu (State Seismological Bureau, Peking) and Wakita (Tokyo University) reported a variety of short-term precursors preceding recent earthquakes greater than magnitude 7 in China and Japan, many of which may be closely related to groundwater (e.g., water level, geochemical and temperature changes in wells, resistivity variations). Sobolev (Institute of Physics of the Earth, Moscow), in a summary of studies in Soviet Central Asia, showed premonitory changes of water levels in wells as far as 650 km from earthquake foci of magnitude 7.

Reviews were presented of the national programs in earthquake prediction in the US, USSR, China and Japan. As described by Mogi (Tokyo University) and Rikitake (Tokyo Institute of Technology) the Japanese program, which began in 1965, involves a closely integrated organization of universities and government agencies monitoring a wide spectrum of geophysical, geological, geodetic and geochemical parameters, with special emphasis on the Izu Peninsula and Tokai district. In addition to the high level of data gathering and research, the Japanese have also made significant

advances in raising public awareness of earthquake hazards and in preparing the public for earthquake emergencies. Participants at the meeting were vividly reminded of the sociological and economic importance of earthquake prediction by a series of Japanese films on the disastrous effects of major earthquakes and a Chinese video-tape showing the destruction caused by the 1976 Tangshan earthquake.

Large earthquakes occur in cycles which are long compared to the instrumental records available of seismicity and related geophysical parameters. The seismic cycle appears even longer when viewed in terms of the relatively short time during which data have been gathered specifically for earthquake prediction purposes. Even within the limitations of existing data, however, the meeting clearly showed that consistent patterns are emerging. As data gathering and interpretation improve, the challenges of the next few years will be to identify the techniques which are the most reliable; to test the significance of predictions based on those methods; and to integrate various techniques into an increasingly reliable procedure for earthquake prediction. □

REVIEW ARTICLE

Biomolecular structure determination by mass spectrometry

Howard R. Morris

Department of Biochemistry, Imperial College of Science and Technology, London SW7 2AZ, UK

Mass spectrometry, conventionally used with smallish molecules (less than 1,000 daltons), has surprisingly emerged as a powerful technique for the determination of the amino acid sequences of proteins and the structure of glycopeptides as well as for the characterization of biologically active materials of unusual structure such as the peptidolipids. Further applications to biomedical problems are foreseen.

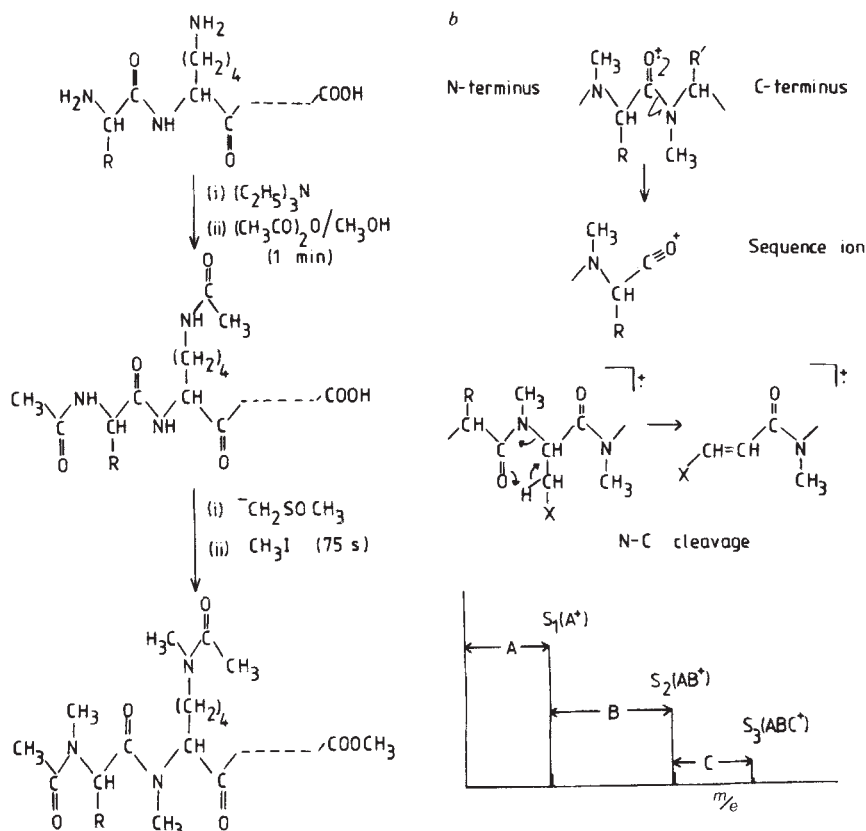
THE past decade has seen a considerable advancement both in the technical capabilities of mass spectrometry and in its application to problems in biochemistry and medicine. Perhaps most surprising, given that most conventional mass spectrometers perform optimally on volatile samples of mass less than 1,000 daltons, has been the successful extension of mass spectrometry to the elucidation of the structures of complex and polar substances such as proteins and carbohydrates. The great power of modern mass spectrometry stems from several fundamental principles peculiar to the technique, not all of which have yet been properly or completely exploited. These are all illustrated in the text that follows but a summary of the concepts will be useful at this stage: (1) sensitivity: mass spectrometry is the most sensitive of all general methods of structure elucidation or quantitation, being routinely applicable to sample sizes of microgrammes or less. In favourable cases picogramme sensitivity has been achieved¹, and progress in the physics of ion generation, mass analysis and detection may eventually lead to the ultimate in sensitivity—generation, analysis and detection of a single ion (detection is already a reality). (2) Specificity: because

the method provides an exact physicochemical measurement (mass with an accuracy of five decimal places for example), its specificity is unequalled by other widely used 'structure probes' such as radioimmunoassay. (3) Sample purity: mass spectrometry has a unique advantage over classical chemical methods or true spectroscopic methods of structure elucidation in that it does not require a pure sample of the compound whose structure is to be determined. It is the intention of this review to illustrate the power and potential of mass spectrometry by examples of its recent role in the determination of protein, peptide, glycopeptide and peptidolipid structure.

Protein sequence analysis

The demand for protein sequence analysis is greater than ever despite recombinant DNA technology, not least because the positive identification of initiator, termination and intron coding sequences demands comparison with amino acid sequence data. The underlying trend in protein sequence analysis is towards the rapid generation of sequence data, not necessarily complete, but sufficient, for example, to combine with crystallographic data to

Fig. 1 *a*, General derivative-forming reactions applicable to 1–50 nmol of protein-derived peptides^{10,11}. To be applicable to all common amino acids, the permethylation step must be restricted with respect to alkylation reaction time to prevent 'salt' formation¹⁰. Arginine-containing peptides require an additional mild hydrazinolysis step before acetylation. This reaction is carried out only on those peptides showing a positive stain test for arginine on the electrophoretogram¹¹ (see 'Strategy'). *b*, The formation of 'sequence ions' and N–C cleavage fragment ions from which the interpretation of the spectrum is made. N–C cleavage is only observed where 'X' corresponds to Phe, Tyr, His, Trp, Asn or Asp¹³. The schematic representation of a mass spectrum shows how the sequence is built up simply by counting mass differences (corresponding to the amino acids A, B and C) between sequence ions S_1 , S_2 and S_3 (ref. 14).



determine tertiary structure, to locate the protein coding in a DNA sequence, or to answer questions on the evolutionary divergence of enzymes. Partial sequence data, following chemical modification of the protein, can also be sufficient to reveal catalytic or binding sites.

It is now 20 years since studies on small synthetic peptides first indicated the possibility of sequence analysis by mass spectrometry^{2,3}, and 10 years since the mass spectrometric analysis of protein-derived peptides and peptide mixtures was first demonstrated⁴. Since then, considerable progress has been made, resulting in combined classical-mass spectrometric attack on total protein sequences, for example on ribitol dehydrogenase⁵ and chloramphenicol transacetylase^{6,7}. Only recently, however, was the first total protein sequence assignment made by mass spectrometry, independent of classical methodology, in work on dihydrofolate reductase^{8,9}. Why has it been so difficult and taken so long to reach this stage? The short answer is that, as already mentioned, the mass spectrometer is not an obvious method of choice for the study of high molecular mass, complex polar molecules. The successful reconciliation of these apparently irreconcilable differences, to produce a methodology that has clear advantages over classical ones (whether manual or automated) has hinged upon the following developments in the areas of derivatization, fragmentation and strategy.

Derivatization and fragmentation

Several types of derivatives are currently in use, the choice depending very much upon the subsequent strategy, for example whether gas chromatography or direct probe insertion is to be used. In most of the protein studies reported to date, samples have been introduced directly via the probe, for which technique the *N*-acetyl, *N,O,S*-permethyl derivative¹⁰ is preferred. Figure 1a summarises the method for obtaining volatile methylated derivatives.

The one feature of the early work that suggested that mass spectrometry might be useful for peptide sequencing was the observation of fragmentation at the peptide bond^{2,3}. This 'sequence ion' formation, first observed for acyl peptide esters, is an even more pronounced fragmentation pathway when the amide bond is methylated, as in the permethylation reaction. Recognition of this important fragmentation pathway is not, however, sufficient for the successful interpretation of the mass spectrum; often the true molecular ion is not present, and other

fragmentation pathways including radical or neutral losses from the molecular ion or other fragment ions¹² (Fig. 1b) must be recognized. The exact nuances of interpretation are too detailed for discussion here, but suffice it to say that current ideas on peptide sequence assignment go well beyond simple recognition of 'sequence ions', often to an understanding and recognition of every signal in the spectrum. This degree of sophistication has been generated by the study of hundreds of peptides in protein sequence analyses so that valuable sequence information can now be obtained from spectra, the interpretation of which might well have been abandoned 10 years ago because of 'ambiguous' assignments.

Strategy

Strategy is crucial to the ultimate success of mass spectrometric determination of biological structures. For example, should one use 'classical' enzymes, like trypsin, for an initial digestion of the protein followed by purification of all the peptides and redigestion of the larger ones? What purification methods will be compatible with mass spectrometry? Is it better to purify the peptides or their derivatives? And what derivatives should be made? Is it best to use high resolution, low resolution, electron impact or chemical ionization? Some of these questions are interrelated; for example, the initial choice of a dipeptidyl aminopeptidase (cathepsin) digest would exclude the production of derivatives by permethylation and would probably lead to gas chromatography-mass spectrometry (GC-MS) separation and identification of dipeptides. Such procedures have been developed¹⁵ but have not yet been applied to proteins of unknown structure. Other GC-MS methods which have been developed include the examination of mixtures of small peptides as trifluoroacetyl permethyl derivatives¹⁶ or as polyamino alcohols^{17,18}.

The most widely applied mass spectrometric method used to date has been low resolution direct probe analysis of peptide mixtures^{4,9}. The great advantage of this approach is that it can deal effectively with mixtures of all sizes of peptide from 2 to 15 residues and above. The ability to sequence unambiguously a mixture of peptides is unique to mass spectrometry¹³, but the significance of this statement is only grasped when one realizes that the isolation and preparation of individual purified peptides is the rate determining step in any classical protein sequencing method; even when using an automated spinning cup sequencer, rarely more than 50-100 amino acids can be placed in sequence

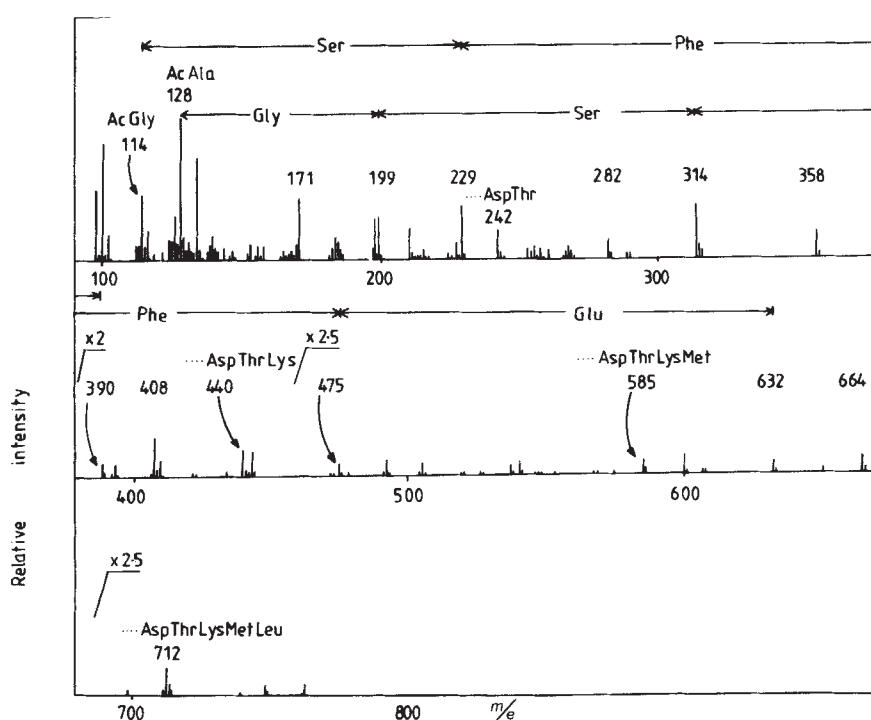


Fig. 2 A typical mass spectrum from the partially purified elastase digest of DHFR, at a source temperature of 240 °C. The assignment of major sequence ions is given, together with a sequence derived from N-C cleavage at aspartic acid. The latter assignment was made by working from high to low mass in the spectrum, looking for amino acid mass differences and expected losses (for example, methanol from Ser or Thr) until *m/e* 242. Since *m/e* 242 loses methanol to *m/e* 210, it must contain either a Ser or Thr. 242 minus serine (115 mass units) is 127—a meaningless N-terminal signal; however, 242 minus threonine (129 m.u.) is 113, which although not an abundant fragment (not well stabilized), is assigned to N-C cleavage at Asp, thus generating the sequence Asp-Thr-Lys-Met-Leu. This, and the other sequences shown, were extended to higher mass at higher source temperatures⁹.

by studying the intact protein. Mixture analysis provides a much needed way of bypassing this rate limiting step in the overall strategy. The principle is one of fractional distillation of the individual peptides from the mixture on the probe tip within the mass spectrometer, using either the probe or ion source heater^{13,19}; the signals associated with any one peptide in the mass spectrum will rise and fall together at different rates to other components in the mixture, depending upon the separation achieved. The result is that each component can normally be sequenced unambiguously, the method being best suited to mixtures of two to five peptides.

To complete the strategy one needs a method of producing peptides of less than about 1,500 molecular mass. Cathepsins can be used¹⁵, but they generate dipeptides, thus destroying the valuable 'overlap' information present in larger peptides. In contrast, trypsin or chymotrypsin may produce fragments too large for the mass spectrometer, and acid hydrolysis leads to loss of amides and other possible complications. It is best, therefore, to use nonspecific proteases, such as thermolysin⁵, elastase¹⁹ or subtilisin²⁰, which produce small peptides (2–15 residues) of ideal size for mass spectrometry.

The overall strategy developed is well illustrated by the study of the sequence of dihydrofolate reductase from methotrexate-resistant *Lactobacillus casei*. Here several digests were utilized, including elastase and subtilisin, and the large cyanogen bromide fragments isolated, to overlap and place the many peptides produced into a final sequence^{8,9}.

Peptide digests are separated by only one cation exchange column step, the effluent being screened analytically by high voltage paper electrophoresis before pooling of peptide mixture fractions, and derivatization. A typical spectrum from the elastase digest, together with its interpretation, is seen in Fig. 2.

Dihydrofolate reductase is the first protein of unknown structure to be fully sequenced by mass spectrometry without the aid of classical methods. Interestingly, with a molecular mass of 18,000 this must be the largest molecule ever to have had its structure determined by mass spectrometry⁹.

New developments in computer automation of the interpretation of mass spectra—at present a skilled task—promise to reduce the time required to sequence a 20,000 dalton protein to about two man months. That, combined with the fact that in experienced hands one needs only 5–30 nmol of a peptide to sequence it, makes mass spectrometry both a complementary approach and a highly competitive alternative to classical techniques of protein sequencing, regardless of whether one chooses the GC-MS method^{16–18} or the direct probe analysis method^{4,5}.

Glycopeptide analysis

Although glycopeptides are increasingly being recognized as an important group of substances, the determination of their structures has proven to be an especially difficult problem. It is difficult in that the structures are complex, demanding information on (1) protein sequence, (2) carbohydrate sequence, (3) nature of protein-carbohydrate linkage, for example O-glycosidic to serine, (4) nature of saccharide-saccharide linkage, for example 1–4, 1–3, and (5) configuration of linkage(s). In seeking such information, mass spectrometry offers the great advantage that it does not suffer from restrictions of compound class as do chemical approaches to the structure; given the normal difficulties encountered with any polar molecule, mass spectrometry can provide information on both the carbohydrate and protein structure from the same experiment. For example, it was necessary to study only two derivatives of AF8, an 'antifreeze' glycopeptide isolated from Antarctic fish blood plasma²¹, to obtain information on each of the first four points above.

The peptide sequence of AF8 was established by studying the electron impact (N-terminal) and chemical ionization (C-terminal) spectra of an acetyl permethyl derivative of the glycopeptide. The spectra showed evidence of heterogeneity in several positions of the 14-residue peptide sequence (Fig. 3a), and the points of attachment of carbohydrate were clearly visible by the assignment of dehydrothreonine, formed by β -

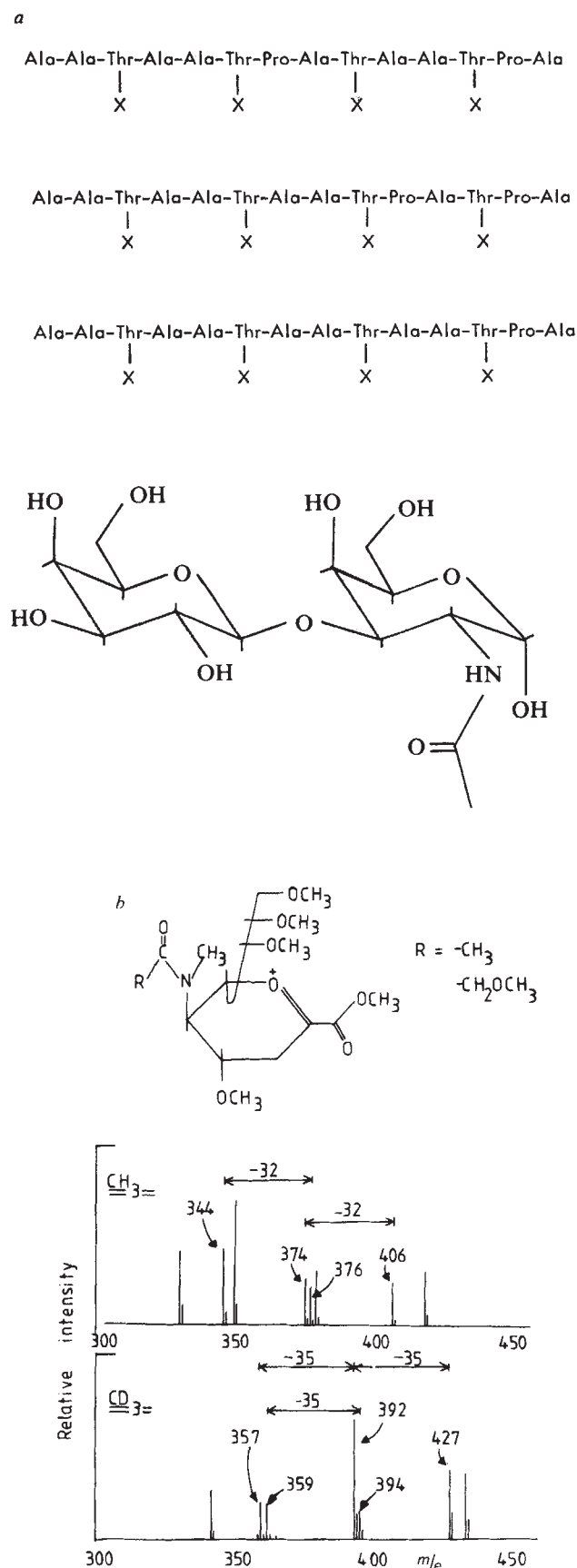


Fig. 3 a, The structure of the glycopeptide AF8 as deduced by electron impact, chemical ionization and field desorption mass spectrometry. b, The partial mass spectrum of a fragment of the glycoprotein prothrombin showing the presence of N-acetyl and N-glycolyl neuraminic acids. Sequence ions were confirmed by the stable isotope experiment shown.

elimination of carbohydrate during addition of the base in the permethylation reaction. Without any necessity for pre-separation, the eliminated permethyl carbohydrate was observed at a different source temperature (fractional distillation) and the sequence assigned as hexosyl-acetamidohexosamine. Interpretation of linkage between saccharides is normally based upon relative abundances of fragment ions which are much more pronounced in pertrimethylsilyl²² rather than permethyl derivatives. In a separate experiment, the carbohydrate was eliminated from the peptide using sodium hydroxide, and again without any purification or separation, a trimethylsilyl derivative was prepared. Comparison of the spectra produced with those of standard 1-4 and 1-3 linked carbohydrates allowed an assignment of a 1-3 linkage in the amino disaccharide (Fig. 3a).

The potential of mass spectrometry in glycopeptide analysis is further illustrated by exploratory work on known structures^{23,24} and on the unknown glycopeptide portion of the blood coagulation zymogen prothrombin²⁵. Here, considerable information has been derived from one of the glycopeptides of molecular weight 4,500 (ref. 25). *N,O*-permethyl derivatives of the intact glycopeptide and its degradation products have been studied to characterize a number of sequences containing up to 10 saccharide units. Interestingly, several of the signals obtained in the spectra are very diagnostic of certain unusual saccharide units, and for example, have given conclusive evidence (via the presence of *m/e* 406 and 374) of the presence of *N*-glycolylneuraminic acid in the prothrombin glycopeptide²⁵ (see Fig. 3b).

Slow-reacting substances

Because of its independence of compound class, mass spectrometry is particularly well suited to the investigation of compounds whose structure is completely unknown. As long as a spectrum can be obtained, it may well suggest the presence of certain units in the structure. More importantly, the complete structure (usually with the exception of stereochemistry) may be deduced by interpretation of the fragmentation pattern in conjunction with the observed molecular weight (from the molecular ion).

A recent example concerns the structure elucidation of a slow-reacting substance (SRS) and its close relative, slow reac-

ting substance of anaphylaxis, SRS-A²⁶, which is a mediator released from lung on antigen challenge, and believed to give rise to allergic bronchospasm (asthma) in man.

The structures of these pharmacologically similar substances have remained unknown since their discovery some 40 years ago²⁷. Chemical clues have been uncovered (for example the probable incorporation of arachidonic acid and cysteine by radio-labelling experiments²⁸), but these have been unconvincing because of the lack of purity of the preparations, and attempts to define the structures of SRSs have relied upon degradative experiments which are inevitably open to question²⁹. Recently, following the earlier development of an HPLC purification procedure for SRS-A and the demonstration of its UV spectrum³⁰, the structures of SRS and SRS-A have been determined, based on the first mass spectra of derivatives of the intact molecules^{31,32}. The SRS spectrum is shown in Fig. 4a. Definitive statements about the structure can now be made because of the exact physicochemical measurements available from the mass spectrum. For example, the molecular weight greatly reduces the number of structural possibilities, particularly since we can define this to five decimal places thus giving the atomic composition of this SRS. We can, however, go much further and use the fragmentation pattern of the molecule to define unambiguously the respective positions of certain structural features in the molecule. A detailed interpretation would not be appropriate here but some guidance is given in Fig. 4. In this way, we can show that SRSs form a novel class of 'peptidolipid'^{31,32} and, in particular, SRS from rat basophil leukaemia cells and SRS-A from guinea pig lung are assigned the structure shown in Fig. 4b, and do not correspond to the recently revised structure of another SRS, termed leukotriene C³³. Human lung SRS-A has previously been shown to co-chromatograph with guinea pig lung SRS-A³⁴, strongly suggesting that this also is the dipeptidyl structure shown in Fig. 4b.

Thus the mass spectrum can give a confidence of structural assignment on very small quantities of material (the above work was accomplished using less than 10 µg total material) in a manner unparalleled by any other physical or chemical technique. Importantly, the mass spectrum also shows what is *not* present in the structure; for example, we can say that neither the

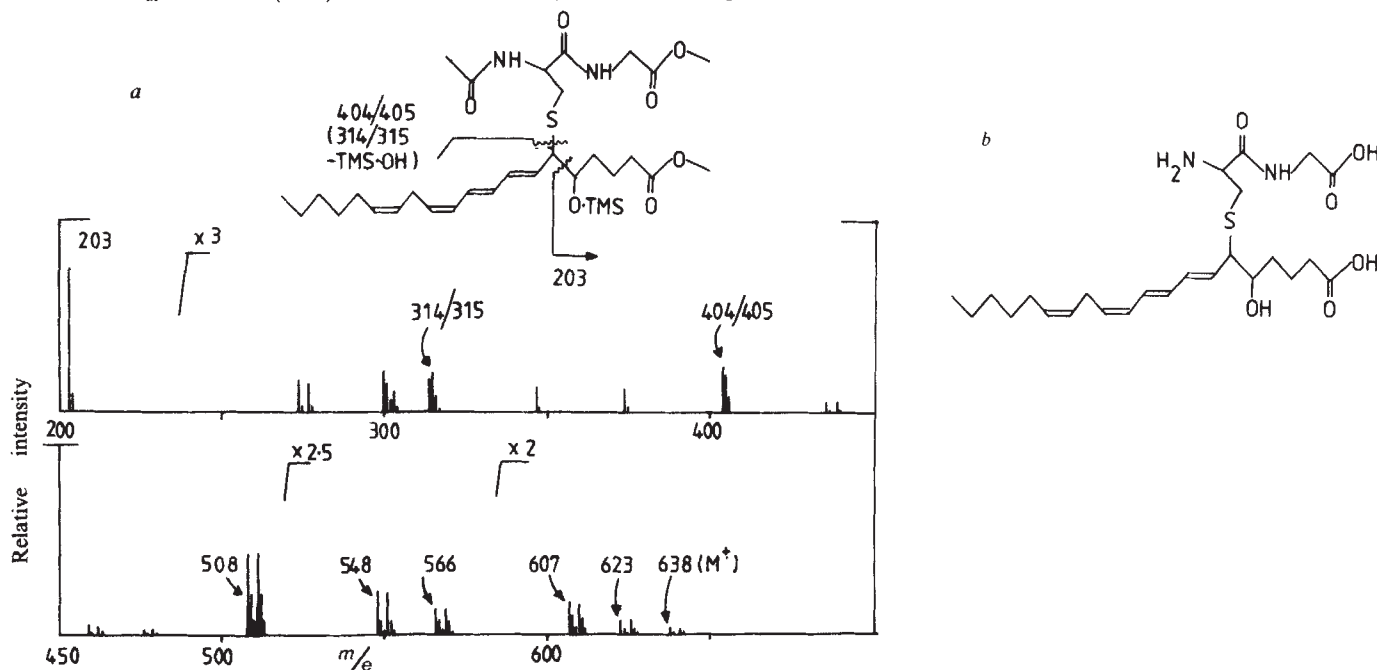
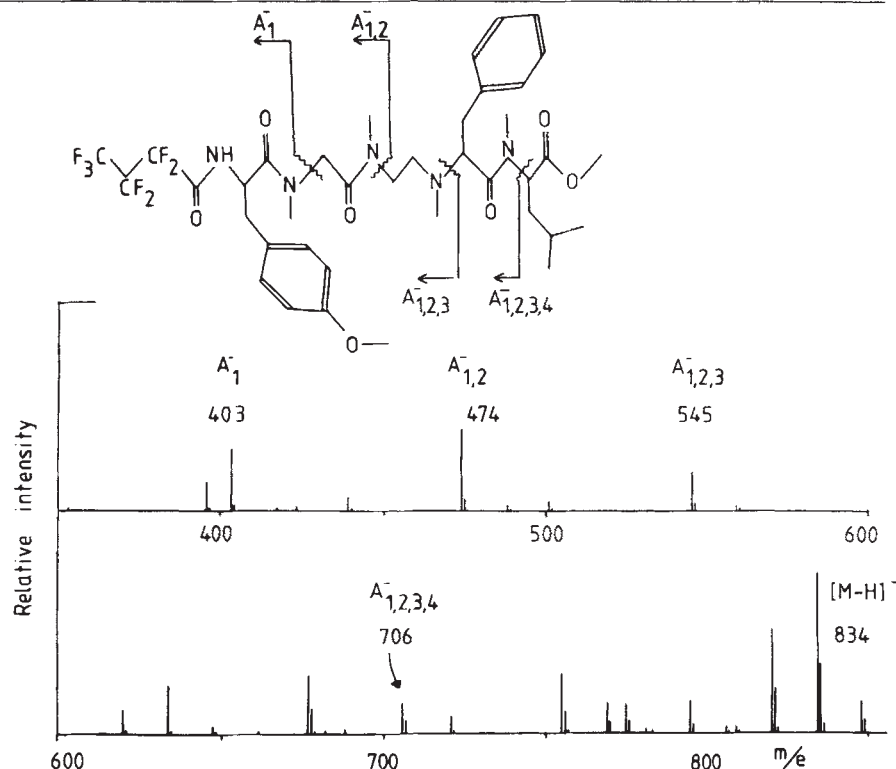


Fig. 4 a, The first mass spectrum of a slow reacting substance (SRS), as the trimethylsilyl ether of the *N*-acetyl, methyl ester derivative³¹. Analytical protein chemical data together with experiments on the destruction of biological activity had indicated the presence of a primary α -amino group in SRS and SRS-A³⁵. The molecule was therefore located and differentiated from background by preparing the 1:1 (²H₃:H₃) acetyl derivative of the unknown. The doublet signals found in the spectrum allowed unambiguous location of those ions corresponding to the unknown SRS, showing a molecular ion at *m/e* 638 and associated losses of $\cdot$ CH₃ and OCH₃ at *m/e* 623 and 607 respectively. Signals at *m/e* 404, 405 and 314, 315 (loss of TMSOH) are diagnostic for the peptide attachment (C₆-S bond) and the arachidonate-derived portion of the molecule. b, The structure of the major SRS³¹ from rat basophil leukaemia cells (RBL-1) and of SRS-A³² from guinea pig lung, deduced from the above spectrum and associated data to be 5-hydroxy-6-cysteinyl-glycyl-7,9,11,14-eicosatetraenoic acid. The mass spectrum does not define stereochemistry.

Fig. 5 The negative chemical ionization (NCI) spectrum of 1 nmol of leucine enkephalin as the *N*-heptafluorobutyl permethyl derivative (A. Buko, D. F. Hunt, M. Judkins and H.R.M., unpublished work). The spectrum shows the increased relative abundance of high mass signals compared with typical EI or CI spectra (no amplification factors in drawing), together with retention of sequence ion information leading to structure (A_1 , $A_{1,2}$ etc.) NCI spectra can be obtained routinely using this type of derivative at the 1 nmol level or less.



RBL-SRS nor the lung SRS-A contain a glycineamide or sulphoxide which would be difficult to discount by chemical or enzymatic methods. Similarly we can say that they do not contain a sulphate moiety, widely expected from the results of destruction of biological activity by aryl sulphatase, reported in the literature.

New and novel amino acids, and 'blocked' proteins

Another important area of biochemistry in which mass spectrometry is playing a significant role is in the study of enzymatic regulation and control in the blood coagulation system.

As with the SRS study above, perhaps the most obvious strength of the mass spectrometric method is in the area of new structures, where no reference compounds exist with which to make a classical interpretation. Just such a situation arose quite surprisingly in 1974 from a sequence study of one of the blood coagulation zymogens, prothrombin. The outcome of this work was the discovery of a new amino acid, γ -carboxyglutamic acid (Gla), and its sequence location in 10 positions in the N-terminal region of prothrombin³⁶⁻³⁸. This amino acid was missed in classical sequencing procedures (in fact it was assigned as ordinary glutamic acid) due to a ready decarboxylation during Edman degradation or hydrolysis in the dansyl procedure.

Since this discovery, there has been considerable interest in the function and occurrence of Gla. In the original work on the identification of Gla, two unique mass spectrometric signals and two unique mass differences were established. These are diagnostic for the new amino acid and the observation of these signals in a mass spectrum allows a definitive assignment not just of the presence of Gla in a peptide but also of its exact location in the sequence³⁸. Thus a recent study of the N-terminal portions of factors X₁ and X₂ (derived by activation of the zymogen factor X) has shown each to contain 12 Gla residues and the two sequences to be identical³⁹.

It should be clear from an understanding of the methods discussed earlier, that the assignment of novel amino acids, such as *N*-methyl lysine, in a peptide sequence reduces to an almost trivial problem. The use of perdeutero-methyl iodide in the permethylation reaction will allow assignment of natural methyl groups simply by counting the mass spectrum (normal lysine would have a mass of 204 mass units, whereas *N*-Me lysine would have a mass of 201 mass units).

Deuterated reagents may also be used to define the presence (or absence) of blocking groups on proteins or peptides. There are now a number of examples in the literature⁴⁰⁻⁴³, including a method for use on proteins⁴⁴, which obviates the necessity for isolation of the blocked N-terminal peptide.

Neurochemistry

Mass spectrometry is playing an increasingly important part in studies on neuropeptides isolated, inevitably in very small quantities, from the central nervous system. Not only the quantity but also the purity may be inadequate for classical structural determination and, furthermore, the confidence in sequence assignment normally associated with protein sequence studies is not available when the sample is not necessarily of protein origin, and, therefore, may have unusual amino acids, unusual linkages or even may not be a peptide at all.

Such a situation occurred in the study of enkephalin, where amino acid analysis, gel column and electrophoretic data did not correlate well with the partial sequence data obtained by the dansyl-Edman method, and an ultraviolet fluorescence measurement raised the further possibility of a 'modified' tryptophan in the molecule⁴⁵. Using the mass spectrometric methods developed for protein sequence analysis, it proved possible to identify two enkephalin pentapeptides, Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu⁴⁶; once the mass spectrometric sequence assignment had been made, and the tryptophan analogue discounted, then the classical data were found to correlate reasonably well with this interpretation.

A number of biologically active neuropeptides have been shown to possess blocked structures, and these pose considerable problems for classical methodology, but fewer problems for mass spectrometry since free peptides would be 'blocked' before study by making derivatives. The difference in 'degree of difficulty' is really quite remarkable here when we consider that the pioneering work on the structure elucidation of thyrotropin releasing hormone (TRH)^{47,48} took some 10 years, and even today the structure of this simple blocked tripeptide would pose very considerable problems for the protein chemist to define, as an unknown. By contrast, the presence of blocking groups actually assists in the mass spectrometric investigation since the formation of a derivative for such a small peptide is no longer necessary. Of course, larger blocked structures still require derivative formation to remove hydrogen bonding and a recent

example of this type of problem is the study of adipokinetic hormone⁴⁹, isolated from locust and responsible for lipid mobilization during flight. The mass spectrum of a permethyl derivative of adipokinetic hormone showed a pyrrolidone carboxylate group (m/e 98 and 126) blocking the N-terminus, and a signal at m/e 428 was interpreted as arising from a C-terminal amide: ... Trp-Gly-Thr-NH₂. The total structure was assigned as a fully blocked decapeptide, PCA-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH₂, and was the first peptide hormone of insect neuroendocrine origin to be completely characterized.

New developments

Several new technical developments promise to improve the analytical value of mass spectrometry still more. Space does not permit a detailed discussion, but briefly the areas covered include ionization techniques giving molecular weight information on underivatized peptides and glycopeptide antibiotics^{50,51}

(field desorption), new approaches to improve sensitivity of detection based upon negative chemical ionization^{1,52}, and developments in magnet technology which have already led to a tripling of the effective mass range of the mass spectrometer at full sensitivity, to over m/e 3,000. The potential of negative chemical ionization in the neurochemical area is illustrated in Fig. 5 showing the spectrum of a Leu-enkephalin derivative, illustrating the derivation of full sequence information at the 1 nmol level.

Thus mass spectrometry is of growing value in the determination of the structure of compounds of biological interest. It is already the method of choice in some cases and is likely to become so in others as the technique develops. In combination with procedures such as high pressure liquid chromatography which can separate picomolar quantities of peptides^{53,54}, mass spectrometry promises to allow the solution of further important chemical and biochemical problems, only just beyond the scope of present technology.

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ARTICLES

Evidence for differential intracellular localization of the *Acanthamoeba* myosin isoenzymes

Hana Gadasi & Edward D. Korn

Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, Building 3, Room B1-20, National Institutes of Health, Bethesda, Maryland 20205

The three myosin isoenzymes of Acanthamoeba castellanii coexist within a single cell and the native molecules probably have the same unusual polypeptide composition as the isolated enzymes. The two myosin I isoenzymes seem to be preferentially localized near the plasma membrane while myosin II seems to be much less concentrated near the plasma membrane than elsewhere in the cytoplasm.

ACTIN and myosin provide the molecular basis for the diverse motility activities of eukaryotic cells¹. Actins from muscle and non-muscle cells are very similar in structure and properties and almost all of the myosins are also structurally similar, containing

a pair of heavy chains of molecular weight (MW) ~200,000 and two pairs of light chains with MWs of about 20,000 and 16,000. Because these non-muscle myosins can also self-assemble into bipolar filaments, it is widely, and not unreasonably, assumed

Fig. 1 Specificity of the reactions of the *Acanthamoeba* myosin heavy chain antibodies with polypeptides of whole amoebae. A centrifugal pellet of intact *Acanthamoeba castellanii* (0.1 ml) was boiled for 20 min in 3 ml sample buffer⁸ containing 1% SDS, 5 mM EGTA, 5 mM EDTA, 0.5 mM phenylmethylsulphonyl fluoride and 0.1 mM pepstatin. Aliquots of 75 μ l each were applied to multiple lanes of 5.6% polyacrylamide slab gels and electrophoresed according to Laemmli⁸. The polypeptides in one lane were stained with Coomassie blue and the polypeptides in the other lanes were transferred to diazobenzyloxymethyl paper as described in the text. The paper was cut into strips corresponding to individual lanes which were then exposed to one of four rabbit IgG fractions (1:50 dilution) and ¹²⁵I-labelled protein A (1×10^6 c.p.m. per lane) as described in the text. Autoradiographs were exposed for 18 h using Kodak no screen X-ray film NS 2T. Lane a, Coomassie blue stained polypeptides; lane b, non-immune IgG; lane c, anti-myosin IA heavy chain IgG; lane d, anti-myosin IB heavy chain IgG; lane e, anti-myosin II heavy chain IgG. The MWs of the radioactive bands in lanes c, d and e are 130,000, 125,000 and 170,000, respectively, relative to standards applied to the same gels, and their electrophoretic mobilities were identical to those of the heavy chains of the corresponding purified myosins on the same gels. Broad smears of apparent labelling (as in lane d) are common but irreproducible occurrences^{9,11}. Occasionally, minor degradation products of the heavy chains were detected.

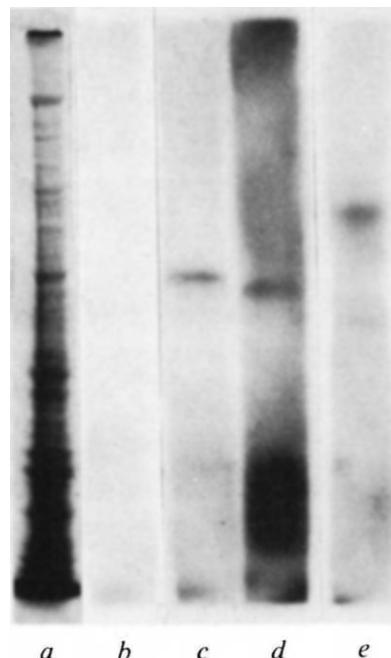
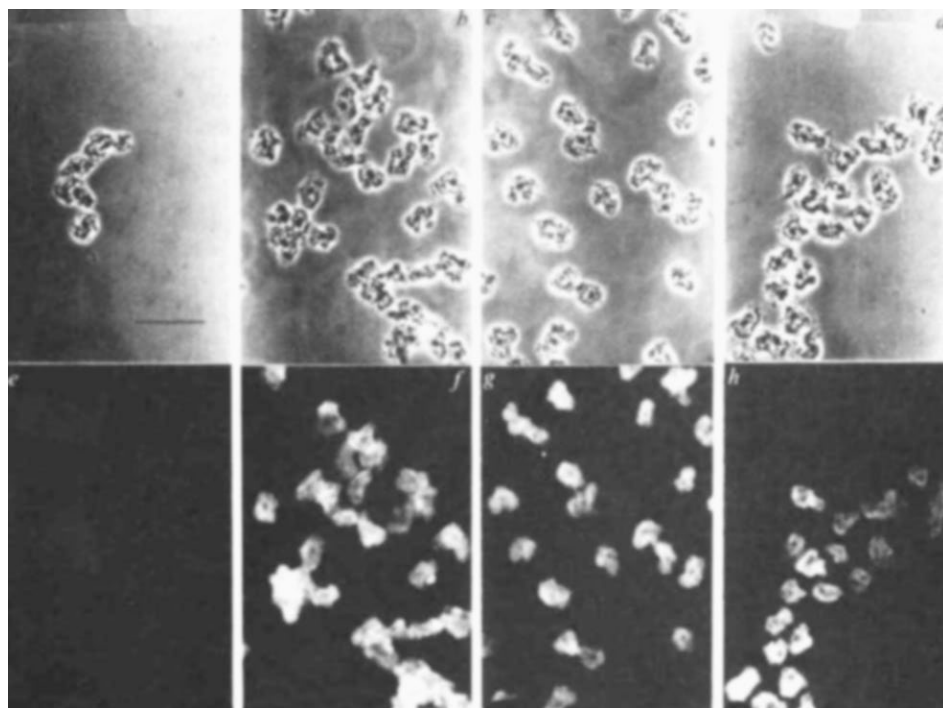


Fig. 2 Indirect immunofluorescence assay of the reaction of *Acanthamoeba castellanii* (Neff strain) with myosin heavy chain antibodies. Cells were allowed to adhere to clean glass slides and were fixed with 5% formaldehyde in phosphate-buffered saline (PBS) for 30 min and then treated with cold (-20°C) acetone for 5 min. They were then sequentially treated with non-immune IgG in PBS, one of the specific antisera (1:200 dilution in PBS containing 1% bovine serum albumin (BSA)) and rhodamine-labelled goat anti-rabbit IgG (1:200 dilution in PBS containing 1% BSA). The cells were washed in 1% BSA in PBS between each treatment. Cells were photographed under phase contrast and fluorescent microscopy at the same magnification. All fluorescent microscopy photographs were made with 30 s exposure and were printed identically. Frames a–d are phase contrast and e–h are fluorescent micrographs of cells reacted with: a, e, non-immune IgG; b, f, antimyosin IA heavy chain IgG; c, g, anti-myosin IB heavy chain IgG; d, h, anti-myosin II heavy chain IgG. Scale bar, 40 μ m.



that a sliding filament mechanism analogous to that responsible for contraction of skeletal muscle is responsible for activities such as amoeboid movement, cell division and phagocytosis in non-muscle cells.

The three myosin isoenzymes isolated from *Acanthamoeba castellanii* (Neff strain) are the only known apparent exceptions. As isolated, *Acanthamoeba* myosins IA and IB²⁻⁵ are both single-headed enzymes of MW about 150,000–180,000. Myosin IA contains a heavy chain of $\sim 130,000$ MW and light chains with MWs of about 17,000 and 14,000, while myosin IB contains a heavy chain of $\sim 125,000$ MW and light chains with MWs of $\sim 27,000$ and 14,000. Both enzymes seem unable to form bipolar filaments². A third myosin isoenzyme, *Acanthamoeba* myosin II, is structurally more conventional^{6,7} and can form bipolar filaments *in vitro*⁷. It is a double-headed molecule with a pair of heavy chains of about 170,000 MW and

two pairs of light chains of about 17,500 and 16,000 MWs, for a native MW of about 400,000. We have shown by peptide mapping⁴ and immunochemical analysis⁵ that the three *Acanthamoeba* myosin isoenzymes are products of different genes.

Because of the unusual structure of two of the *Acanthamoeba* myosin isoenzymes and because no other non-muscle cell has yet been shown to contain more than one species of myosin, we thought it important to establish: (1) if the isolated enzymes are in their native forms; (2) if the isoenzymes coexist in a single cell; and (3) whether the myosin isoenzymes are differentially localized within the cell. We have now obtained affirmative answers to all three questions through the use of specific antibodies raised against the isolated heavy chains of the three myosin isoenzymes. The differential localizations of the isoenzymes raise the interesting possibility that the different myosin species serve specific and different motility functions, and the unusual

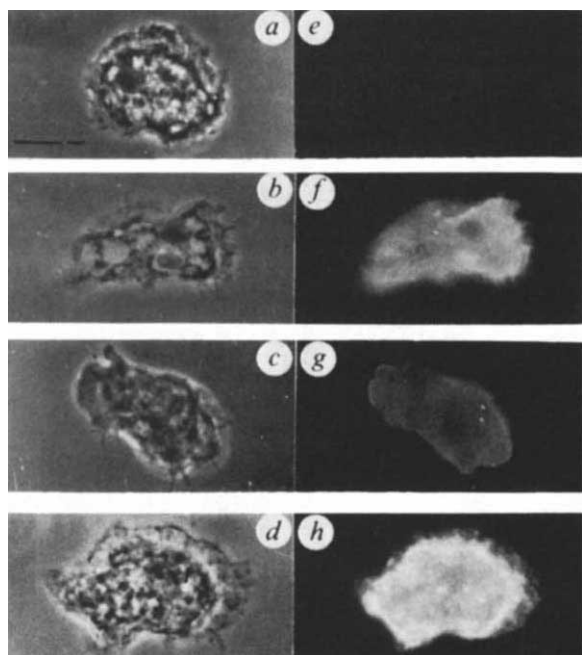


Fig. 3 Differential localization of myosin isoenzymes by indirect immunofluorescence. This experiment was identical to that described in Fig. 2. Frames *a*–*d*, phase contrast; *e*–*h*, fluorescence; *a*, *e*, non-immune IgG; *b*, *f*, anti-myosin IA heavy chain IgG; *c*, *g*, anti-myosin IB heavy chain IgG; *d*, *h*, anti-myosin II heavy chain IgG. Scale bar, 10 μ m.

physical properties of the myosin I isoenzymes suggest that some of these functions might not involve the classical sliding filament mechanism.

Preparation of heavy chain antibodies

Previously⁵, we had raised antibodies against the isolated myosins. Although the enzymes were highly purified, they were contaminated by small amounts of other amoeba proteins, which might also have elicited sufficient antibodies to make those antisera inappropriate for cytochemistry. Therefore, monospecific myosin heavy chain antibodies were prepared as follows. Highly enriched preparations of *Acanthamoeba* myosins IA, IB and II were obtained by direct immunoprecipitation with the appropriate antibodies previously raised against the isolated enzymes. The immunoprecipitates were separated by electrophoresis⁸ on SDS-5.6% polyacrylamide slab gels and the gels were stained lightly with Coomassie blue. In each case⁵, only a single major band, corresponding in mobility to the 130,000, 125,000 and 170,000-MW heavy chains of myosin IA, IB and II, respectively, was present above the dye front. These bands were cut out, washed, sonicated and injected into rabbits to elicit specific antibodies against each of the three heavy chains. Prepared in this way, the only possible contaminating antigens from the amoebae would be molecules which were precipitated from the amoeba extract by the antibody raised against the whole myosin and which also co-migrated electrophoretically with the myosin heavy chain. Such coincidence is unlikely. Immune and non-immune sera were precipitated with 45% ammonium sulphate and the IgG fractions were dissolved in phosphate buffered saline.

Specificity of heavy chain antibodies

We tested, in the same experiment, both the specificity of the three myosin heavy chain antisera and the possibility that higher molecular weight forms of the heavy chains might exist in the amoebae. Whole amoebae were boiled in 1% SDS containing several inhibitors of proteolytic enzymes and multiple identical samples were separated by SDS-polyacrylamide slab gel elec-

trophoresis. One lane was excised and stained for polypeptides with Coomassie blue while the polypeptides in the duplicate lanes were transferred to diazobenzyloxymethyl paper⁹. The paper was cut into individual lanes which were incubated with a large excess of either non-immune sera or one of the specific myosin heavy chain antibodies followed by ¹²⁵I-protein A (ref. 10) and the positions of the bound antibodies were located by autoradiography.

The antibodies raised against the isolated heavy chains of myosin IA, IB and II reacted exclusively (Fig. 1) with polypeptides of molecular weights corresponding exactly to those of the heavy chains of the purified enzymes: 130,000, 125,000 and 170,000, respectively. Similar results have been obtained in 15 to 20 experiments including some in which the polypeptides were reacted with the antisera on the polyacrylamide gel without transfer to the paper. Occasionally, minor degradation products of the myosin heavy chains were detected but never a polypeptide of higher molecular weight than that of the heavy chains of the purified myosins. Degradation products were never detected when extracts of amoeba, rather than whole cells, were analysed, probably because the lysosomal proteases were removed before addition of SDS. To demonstrate that, if it were present in the amoebae, a polypeptide of the size typical of the heavy chains of other myosins would have been detected, we mixed pure chicken pectoralis muscle myosin with a sample of the amoebae just before they were added to the SDS solution. The undegraded 200,000-MW heavy chain of the chicken myosin was readily detected using its specific antibody. These experiments, therefore, provide strong evidence that the cells do not contain immunologically cross-reactive higher molecular weight forms of the heavy chains of *Acanthamoeba* myosins IA, IB and II.

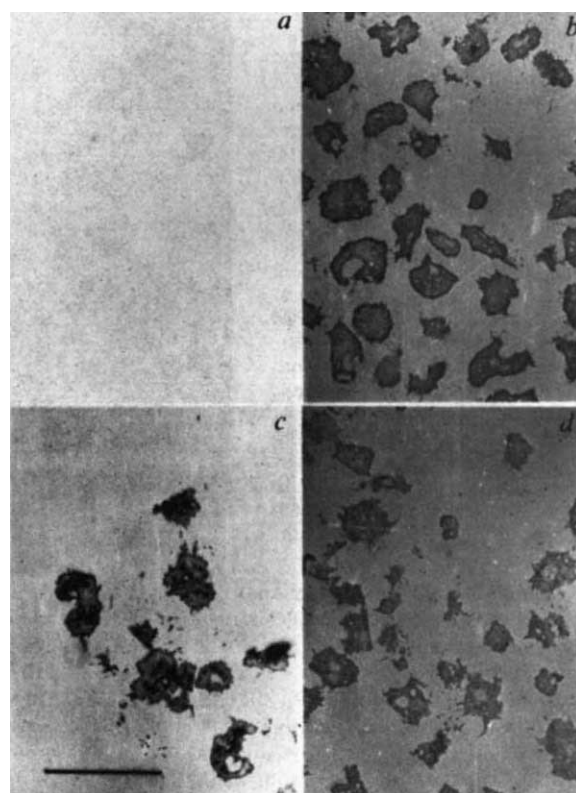
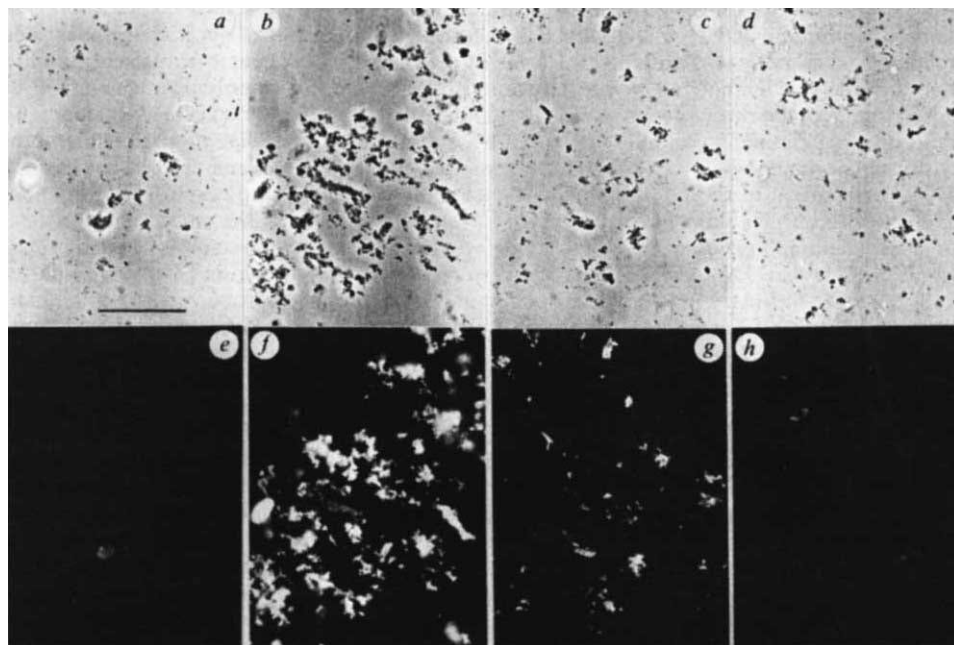


Fig. 4 Immunocytochemical localization of myosin isoenzymes. Sections (1 μ m) were reacted with specific antisera (1:250 dilution) and subsequently with the peroxidase-anti-peroxidase system as described in the text. All micrographs were photographed and printed under identical exposures. Frame *a*, non-immune IgG; *b*, anti-myosin IA heavy chain IgG; *c*, anti-myosin IB heavy chain IgG; *d*, anti-myosin II heavy chain IgG. Scale bar, 40 μ m.

Fig. 5 Indirect immunofluorescence assay of isolated plasma membranes. Suspensions of purified plasma membranes¹⁶ were fixed for 30 min with 3.7% formaldehyde and then reacted with specific antisera (1:50 dilution) followed by rhodamine-labelled goat anti-rabbit IgG (1:200 dilution). The membranes were then applied to slides and the same fields were photographed under phase contrast (a–d) and fluorescence (e–h). Exposure time was 20 s and all the fluorescent micrographs were printed under identical exposures. Non-immune IgG (a, e); anti-myosin IA heavy chain IgG (b, f); anti-myosin IB heavy chain IgG (c, g); anti-myosin II heavy chain IgG (d, h). Scale bar, 40 μ m.



Myosin isoenzymes occur in the same cells

The data shown in Fig. 1 demonstrate that the antibodies to the myosin heavy chains could be used in cytochemical experiments to detect myosin II and the two myosin I isoenzymes in the presence of each other and without interference by other amoeba proteins. Although the antibodies to myosin IA and IB heavy chains also did not seem to cross-react (Fig. 1, lanes c, d), we were not certain that we could selectively detect myosin IA or IB in the presence of the other because previous antisera to the purified intact myosins IA and IB did cross-react⁵ and because direct inspection of some of the autoradiograms obtained with antibody to myosin IA heavy chain indicated slight reaction with the 125,000-MW heavy chain of myosin IB.

We used the indirect immunofluorescence method to determine if each of the *Acanthamoeba* myosin isoenzymes occurs in every cell. Amoebae were allowed to crawl on clean glass slides¹². The cells that adhered were fixed with formaldehyde and made permeable to antibodies by treatment with cold acetone. Cells were then exposed to antibodies to either myosin IA heavy chain, myosin IB heavy chain or myosin II heavy chain followed by rhodamine-labelled goat anti-rabbit immunoglobulin¹³. The same fields of cells were photographed at low magnification ($\times 160$) under phase contrast (Fig. 2a–d) or fluorescence (Fig. 2e–h) microscopy. As every cell on all of the slides was fluorescent irrespective of which of the three specific antisera was used, all the cells must have contained myosin II and at least one, but probably both, of the myosin I isoenzymes.

Intracellular localization of myosin isoenzymes

In Fig. 2, the fluorescent image of every cell exposed to the antibodies to myosin IA and myosin IB heavy chains is the same size as the corresponding phase contrast image (Fig. 2, f compared with b and g compared with c), whereas the fluorescent image of every cell exposed to the antibodies to myosin II heavy chain is smaller than the corresponding phase contrast image (Fig. 2, h compared with d). The reason for this difference can be seen at higher magnification, as in Fig. 3. Antibodies to myosin IA and IB heavy chains were bound more intensely at, or near, the plasma membrane than elsewhere in the cell (Fig. 3f, g) and, therefore, the fluorescent images of the cells have the same overall dimensions as the phase contrast images (Fig. 3b, c). On the other hand, the periphery of the cell and the cytoplasmic region adjacent to the plasma membrane reacted much less well with antibodies to myosin II heavy chain

than did the rest of the cytoplasm (Fig. 3h). As a result, the fluorescent image of the cell is smaller than the corresponding phase contrast image (Fig. 3d). Therefore, the myosin I isoenzymes seem to be more concentrated at or near the plasma membrane, from where myosin II is mostly excluded. No reaction was detected with any antisera unless the cells were made permeable; none of the antigenic sites, therefore, is on the external surface of the amoeba.

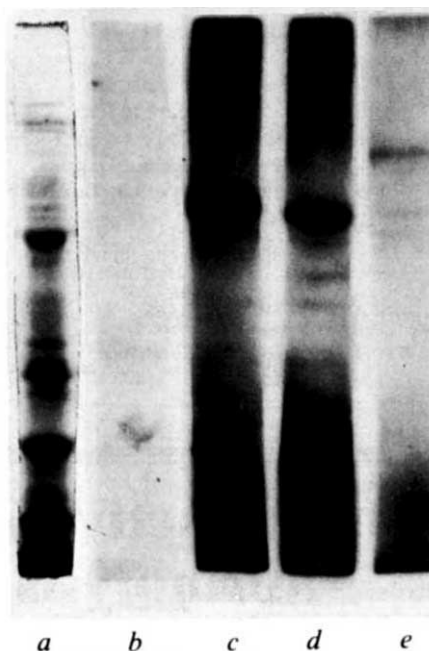


Fig. 6 Reaction of *Acanthamoeba* myosin heavy chain antibodies with plasma membrane polypeptides. Isolated plasma membranes of *Acanthamoeba castellanii* (Neff) were analysed identically as described for whole cells except that 7.5% polyacrylamide gels were used. Lane a, Coomassie blue stained polypeptides; lane b, non-immune IgG; lane c, anti-myosin IA heavy chain IgG; lane d, anti-myosin IB heavy chain. The autoradiograms (lanes b and d) were overexposed to detect the very low reaction with myosin II heavy chain and, therefore, minor degradation products of some of the heavy chains and nonspecific smears, may be detected in addition to the major bands. The major bands in lanes c, d and e are at the exact positions of the heavy chains of the respective purified myosins which were applied as standards to the same slab gel.

Additional evidence for the differential localization of *Acanthamoeba* myosins I and II was obtained by an independent immunocytochemical method. Transverse sections of cells fixed in glutaraldehyde and embedded in polyethylene glycol¹⁴ were exposed to one of the three specific antisera and incubated with goat anti-rabbit immunoglobulin followed by rabbit anti-peroxidase immunoglobulin coupled to horseradish peroxidase¹⁵. The position of the bound peroxidase was determined cytochemically by incubation for 30 min with the peroxidase substrates, diaminobenzidine and hydrogen peroxide. When antibodies to myosin IA and IB heavy chains were used (Fig. 4b, c), the cell boundary was intensely stained with brown reaction product, and the cytoplasm was diffusely stained, the antibodies to myosin II heavy chain resulting in only diffuse staining of the cytoplasm (Fig. 4d).

Myosins of isolated plasma membranes

By indirect immunofluorescence, isolated plasma membranes¹⁶ were found to react with antibodies to myosin IA and IB heavy chains (Fig. 5f, g) but not with antibodies to myosin II heavy chain (Fig. 5h). Myosin IA and myosin IB were also concentrated in the isolated plasma membranes as determined by specific staining of SDS-polyacrylamide electrophoretic gels. The intensity of the radioactive labelling of the 130,000- and 125,000-MW polypeptides of myosin IA and IB was very much greater than the barely detectable labelling of the 170,000-MW myosin II heavy chain (Fig. 6), in contrast to the essentially equivalent labelling of the three polypeptides in electrophoretic gels of the whole amoebae (Fig. 1). Also, the absolute intensity of labelling of the 130,000- and 125,000-MW peptides was very much greater for the plasma membranes (Fig. 6) than for whole amoebae (Fig. 1), although the same amounts of protein were applied. Both myosin I isoenzymes, therefore, were enriched in the isolated plasma membranes in comparison with myosin II and also relative to their own concentrations in the whole amoebae.

Conclusions

From the data in this and previous papers from our laboratory, we conclude that *Acanthamoeba castellanii* (Neff) contains three myosin isoenzymes: single-headed myosins IA and IB and double-headed myosin II having heavy chains of the unusually

low MWs of 130,000, 125,000 and 170,000, respectively. The myosin I isoenzymes seem to be preferentially localized at or near the plasma membrane while myosin II seems to occur mainly, if not exclusively, deeper in the cytoplasm. We do not know if the myosin I isoenzymes are bound directly to the isolated plasma membrane or are associated with the actin filaments that lie immediately under the plasma membrane¹⁷ and co-purify with the plasma membranes^{18,19}.

We must consider, of course, the possibility that the experimental data do not accurately reflect the distributions of the myosin isoenzymes in the living amoebae. When cells are fixed, soluble myosins might precipitate, or attach to actin filaments, in places where they do not normally occur, or myosins might be lost from cells during the subsequent steps of the cytochemical procedures. Similarly, myosin isoenzymes might differentially redistribute during the isolation of plasma membranes.

However, although none of these possible sources of errors has been unequivocally eliminated, we think it unlikely that three entirely independent methods, fixation by formaldehyde, fixation by glutaraldehyde and isolation of plasma membranes, would have given the same misleading result. Moreover, myosin II is larger and less soluble than myosin IA and IB and all three purified myosins bind tightly to F-actin, which greatly stimulates their Mg^{2+} -ATPase activities. There is no reason to suppose, therefore, that myosin II would be preferentially extracted from, or that myosins IA and IB would selectively and artefactually bind to, just that region of the cell which is particularly enriched in actin filaments. Nonetheless, it is important that the observations in this paper be confirmed by independent methods.

Finally, if the myosin I and II isoenzymes are, as we suggest, differentially localized, it is likely that they function in different cellular activities with the myosin I isoenzymes being involved in processes, possibly such as endocytosis, that occur at or near the cell surface. Because of the unusual physical properties of myosin IA and IB, their functions may involve actomyosin interactions of a type not previously proposed.

We thank Dr Susan Lowey for chicken pectoralis muscle myosin and its antibody, Dr Blair Bowers for guidance in the immunocytochemical procedures, Dr Michael Bustin for help with the immunofluorescence studies, and Mr Thomas Olszewski and Mrs Margaret Clark for technical assistance.

Received 19 February; accepted 9 May 1980.

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Surface activation of blood coagulation, fibrinolysis and kinin formation

Ronald L. Heimark, Kotoku Kurachi, Kazuo Fujikawa & Earl W. Davie

Department of Biochemistry, University of Washington, Seattle, Washington 98195

The activation of plasma prekallikrein by single-chain factor XII has been studied in the presence of high molecular weight kininogen and kaolin. The data indicate that factor XII can initiate blood coagulation, fibrinolysis or kinin generation in the presence of kaolin and does so by converting prekallikrein to kallikrein. An enzyme cascade is then generated leading to the formation of fibrin, plasmin or bradykinin in three closely related physiological events.

THE initiation of the contact phase of blood coagulation by a surface, such as glass or kaolin, leads to an enzyme cascade resulting in fibrin formation¹⁻³. A similar enzyme cascade occurs

during the formation of plasmin in the surface-activated fibrinolytic system and the generation of kinin in the presence of a surface.

The molecular basis for the initiation of these three closely related processes has been difficult to explain as two of the proteins involved in the surface activation reaction, factor XII (Hageman factor, ref. 4) and prekallikrein^{5,6} (Fletcher factor), are present in plasma as zymogens to serine proteases. Furthermore, these two proteins have an unusual relationship to each other in that factor XII_a converts prekallikrein to kallikrein⁶⁻¹⁰, and kallikrein converts factor XII to factor XII_a (refs 9, 11-16). Thus, it has been difficult to determine whether factor XII_a, kallikrein or some other protein is responsible for initiating these cascade reactions. A third plasma protein, high molecular weight kininogen¹⁷⁻²⁰ (HMW kininogen), stimulates the activation of prekallikrein by factor XII_a and the activation of factor XII by kallikrein²¹⁻²⁶. HMW kininogen seems to function as a cofactor in these reactions.

The role of factor XII and factor XII_a

Considerable attention has been directed towards factor XII as the principal protein involved in the surface activation of blood coagulation, fibrinolysis and kinin formation. Bovine and human factor XII are single-chain glycoproteins with molecular weights of about 74,000 (refs 27-29). The bovine preparation has no detectable esterase or amidase activity in the absence or presence of a surface, such as kaolin, dextran sulphate, or sulphatide^{26,30}. Furthermore, it shows very little reactivity towards serine protease inhibitors, such as diisopropyl fluorophosphate or antithrombin III and heparin, in the absence or presence of a surface. Bovine factor XII_a is composed of a heavy chain (MW 46,000) and a light chain (MW 28,000), and these two chains are held together by a disulphide bond(s)³⁰. The two-chain protein has substantial esterase and amidase activity and readily activates prekallikrein³¹ and factor XI_a (ref. 32) by the cleavage of a specific internal peptide bond(s) in each of these proteins. Bovine factor XII_a is also readily inhibited by diisopropyl fluorophosphate or antithrombin III and heparin³⁰. In contrast, single-chain human factor XII possesses amidase activity when previously exposed to ellagic acid-Sepharose³³. Human factor XII, however, reacts about 600 times more slowly with diisopropyl fluorophosphate than factor XII_a in the presence or absence of kaolin³⁴. Accordingly, it has been suggested that factor XII may participate in the initiation of blood coagulation as a weakly active zymogen³⁴. In whole plasma, radiolabelled human factor XII was shown to be converted to factor XII_a during the coagulation process³⁵. It was not established, however, whether the activation of factor XII was an initial or a secondary event in the contact activation reaction. Furthermore, it has not been clear from the various studies on human and bovine factor XII whether the single-chain molecule or the two-chain factor XII_a participates as a catalyst in the initial reactions involving prekallikrein and HMW kininogen.

Involvement of factor XII in factor XI activation

Recently, Saito²⁴ reported the activation of human factor XI (plasma thromboplastin antecedent) by factor XII in the presence of HMW kininogen and ellagic acid. No evidence was obtained to indicate that human factor XII was converted into lower molecular weight fragments during the reaction. We have studied the activation of bovine factor XI by single-chain bovine factor XII in the presence of sulphatide, dextran sulphate or kaolin plus HMW kininogen³². In the latter experiments, factor XI (MW 124,000) was converted to factor XI_a (MW 124,000) by the cleavage of an internal peptide bond in each of the two nearly identical chains of the precursor molecule. The bovine factor XII was converted to factor XII_a in the latter portion of this reaction, however, by the newly generated factor XI_a (ref. 32). Homogeneous preparations of bovine factor XI (ref. 36), factor XII (ref. 27) and HMW kininogen²⁶ were used in these studies. The activation of factor XI by factor XII provided direct evidence to support the concept that single-chain factor XII can act as a catalyst, but only in the presence of a highly specific substrate and a negatively charged surface.

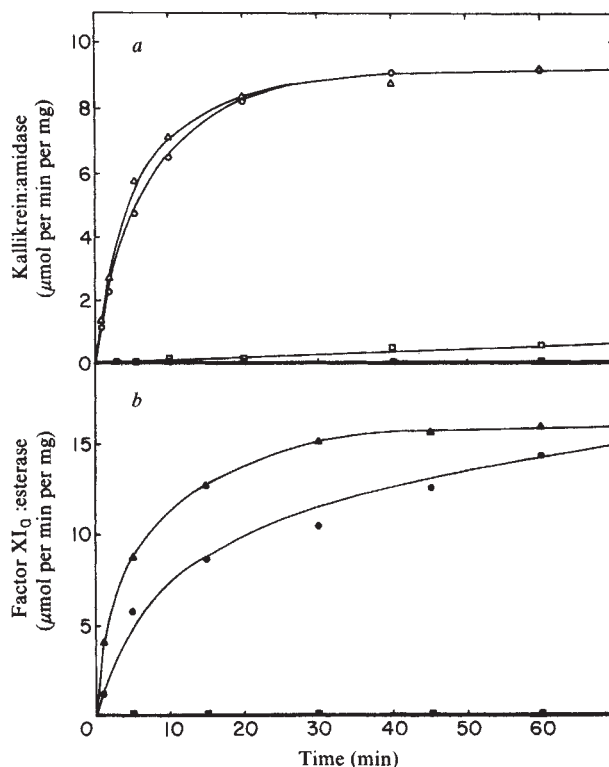


Fig. 1 Time course for the activation of *a*, plasma prekallikrein and *b*, factor XI by factor XII or factor XII_a in the presence of HMW kininogen and kaolin. Factor XII or factor XII_a (0.14 μg) was preincubated with HMW kininogen (0.4 μg) and kaolin (20 μg) for 5 min at 37 °C in 0.05 M Tris-HCl, pH 7.5, containing 0.1 M NaCl. Reactions were initiated by the addition of prekallikrein (9 μg) or factor XI (9 μg). Incubations were carried out in siliconized glass tubes in a final volume of 0.1 ml. Aliquots (10 μl) were removed at various times and assayed for kallikrein or factor XI_a. In the assay for kallikrein: amidase activity, the samples were added to 0.99 ml of 0.05 M Tris-HCl, pH 8.0, containing 0.15 M NaCl, 10 mg per ml polybrene, 50 μg lima bean trypsin inhibitor, and 0.1 ml of 1.5 mM benzoyl-Pro-Phe-Arg-p-nitroanilide. The initial rate of amide hydrolysis was followed at 37 °C by the change in absorbance at 405 nm using a Gilford recording spectrophotometer. For the factor XI_a: esterase activity, aliquots were added to a solution of factor XII antibody to stop the reaction and hydrolysis of tosyl-L-arginine-³H-methyl ester was followed in a Beckman scintillation counter (SL-100C) as previously described³². The coagulation proteins were prepared from bovine plasma and all were homogeneous by SDS-polyacrylamide gel electrophoresis. Protein concentrations were determined by absorption at 280 nm using an $E_{280}^{1\%}$ of 10.9 for prekallikrein³⁷, 12.6 for factor XI (ref. 36), 14.2 for factor XII (ref. 27) and the same value for factor XII_a. Their specific activities in a clotting assay with their respective deficient plasmas were as follows: factor XII (180 u per mg), factor XII_a (280 u per mg), HMW kininogen (11 u per mg), plasma prekallikrein (60 u per mg), and factor XI (280 u per mg). Factor XII was treated with diisopropyl fluorophosphate (1 mM) at 37 °C for 60 min to remove any traces of factor XII_a, and the diisopropyl fluorophosphate was removed by dialysis against the reaction buffer. *a*: ○, prekallikrein plus factor XII, HMW kininogen and kaolin; △, prekallikrein plus factor XII_a, HMW kininogen and kaolin; ■, prekallikrein plus HMW kininogen and kaolin; □, prekallikrein plus factor XII. *b*: ●, factor XI plus factor XII, HMW kininogen and kaolin; ▲, factor XI plus factor XII_a, HMW kininogen and kaolin; ■, factor XI plus HMW kininogen and kaolin.

Activation of prekallikrein by factor XII and/or factor XII_a

In the present report, we describe the activation of bovine plasma prekallikrein by single-chain factor XII as well as factor XII_a in the presence of HMW kininogen and kaolin. In these experiments, a homogeneous preparation of single-chain plasma prekallikrein (MW 82,000)³⁷ was used as substrate. This preparation of prekallikrein contained no detectable kallikrein or factor XII activity. Bovine factor XII or factor XII_a was added to optimum amounts of HMW kininogen and kaolin, and the activation reaction was initiated by the addition of prekallikrein. The factor XII or factor XII_a-to-prekallikrein weight ratio was 1:65. In these conditions, prekallikrein was rapidly converted to

kallikrein (Fig. 1a). The generation of kallikrein was measured by its amidase activity towards benzoyl-L-prolyl-L-phenylalanyl-L-arginine-*p*-nitroanilide. The initial rate of kallikrein generation was almost the same with either factor XII (open circles) or factor XII_a (open triangles). No lag phase or sigmoidal curve was observed for the activation of prekallikrein by factor XII. A lag phase would be expected, however, if the conversion of factor XII to factor XII_a were essential in this reaction. No activation of prekallikrein occurred in the absence of factor XII or factor XII_a (solid squares). A small amount of activation occurred in the absence of kaolin (open squares). When prekallikrein was omitted from the reaction, no amidase activity was generated.

To test for the possibility of an extremely rapid conversion of factor XII to factor XII_a by kallikrein early in the activation reaction, the conversion of prekallikrein to kallikrein was studied using ³H-labelled factor XII as the enzyme. These experiments are important because kallikrein readily converts factor XII to factor XII_a, and this reaction could greatly reduce any lag phase in the reaction if the activation of prekallikrein were catalysed only by factor XII_a. The ³H-factor XII was prepared by reductive alkylation by the procedure of Means and Feeney³⁸ using ³H-NaBH₄ and formaldehyde. The ³H-factor XII had the same clotting activity as the unlabelled protein and contained approximately 143,000 c.p.m. per µg. Furthermore, when a catalytic amount of kallikrein was used to activate ³H-factor XII in the presence of kaolin, the generation of factor XII_a esterase activity occurred in parallel with the disappearance of single-chain factor XII and the appearance of two-chain factor XII_a. This was shown by SDS polyacrylamide gel electrophoresis of the reaction products in reducing conditions (data not shown).

Activation of prekallikrein by factor XII

A time course for the activation of prekallikrein by ³H-factor XII in the presence of HMW kininogen and kaolin is shown in Fig. 2a. The rate of formation of kallikrein (open circles) was very similar to that shown in Fig. 1a, and no lag phase was observed. The activation of prekallikrein was also followed by SDS-polyacrylamide gel electrophoresis on a slab gel using the method of Laemmli³⁹. In these experiments, samples were removed at various times during the activation reaction by factor XII and examined in the presence and absence of 2-mercaptoethanol. In the absence of reducing agent, prekallikrein appeared as a band of MW 82,000. This indicated that little, if any, change in the molecular weight of prekallikrein occurred during its conversion to kallikrein. A difference in the SDS-polyacrylamide gel electrophoresis pattern of the prekallikrein was observed, however, after reduction of the samples with 2-mercaptoethanol. At zero time, prekallikrein appeared as a 82,000-MW band. During the first few minutes of the activation reaction, three new, faster-moving bands appeared, including a heavy chain (apparent MW 52,000) and a light chain (apparent MW 38,000 or 33,000). The appearance of these bands as measured by densitometry (Fig. 2a, open triangles) occurred in parallel with the formation of kallikrein as measured by its amidase activity (Fig. 2a, open circles). These data indicate that bovine prekallikrein was converted to kallikrein by the hydrolysis of an internal peptide bond in the single-chain precursor molecule in the presence of HMW kininogen and kaolin. The cleavage of bovine prekallikrein by factor XII is very similar to that reported earlier for human prekallikrein activated by human factor XII_a fragments (ref. 10). The SDS-polyacrylamide slab gel was then prepared for fluorography⁴⁰ and the amount of radioactivity present in ³H-factor XII was determined. At zero time, the radiolabelled protein migrated as a single band (approximate MW 74,000) on SDS-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol. As the ³H-factor XII was converted to ³H-factor XII_a by the newly generated kallikrein, the ³H-factor XII_a appeared as a heavy chain (MW 46,000) and a light chain (MW 28,000) in the presence of 2-mercaptoethanol. After 2.5 min, 35% of the

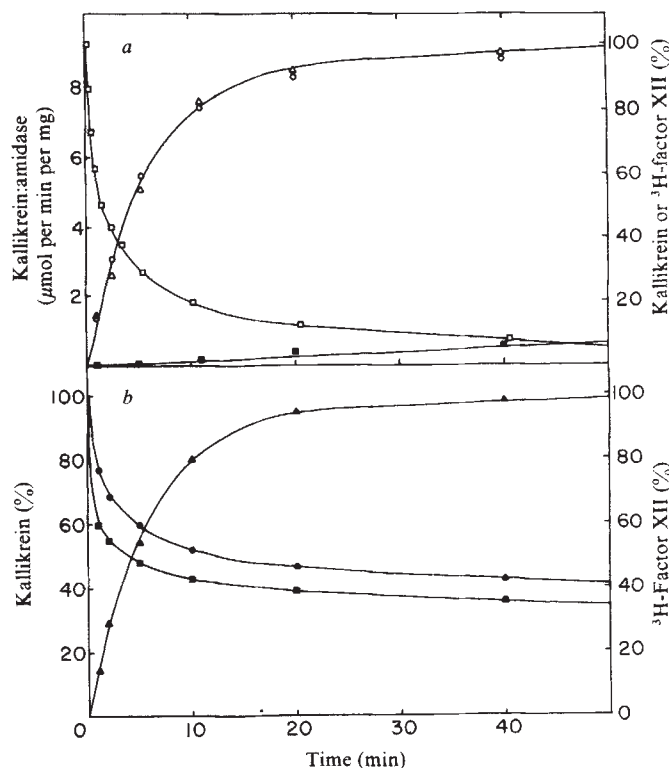


Fig. 2 *a*, Time course for the activation of plasma prekallikrein by ³H-factor XII in the presence of HMW kininogen and kaolin. *b*, The reaction in the presence of RVV inhibitor II, a serine protease inhibitor from Russell's viper venom. ³H-factor XII (0.84 µg) was preincubated with HMW kininogen (2.4 µg) and kaolin (120 µg) for 5 min at 37 °C in 0.05 M Tris-HCl, pH 7.5, containing 0.1 M NaCl. Reactions were then initiated by the addition of prekallikrein (55 µg) to give a final volume of 0.6 ml. Aliquots (10 µl) were removed at various times and assayed for kallikrein:amidase activity for the experiments shown in *a*. Samples (50 µl) were also subjected to SDS-polyacrylamide gel electrophoresis in the presence of 5% 2-mercaptoethanol. The samples for electrophoresis were immediately added to sample buffer, boiled for 1 min, and then analysed on a 10% polyacrylamide slab gel. After staining (Coomassie brilliant blue R) and destaining, the gel was scanned with a Joyce-Loebl densitometer and the amount of kallikrein was determined from the tracings. To determine the amount of ³H-factor XII, the gel was prepared for fluorography⁴⁰. After developing the fluorogram, the band of ³H-factor XII was sliced from the dried gel and the amount of radioactivity was determined by dissolving the gel slices in 30% H₂O₂ (99 vol:1 vol NH₄OH) followed by counting in Aquasol (NEN). The counting efficiency was 30%. *a*: ○, generation of kallikrein:amidase activity in the complete reaction mixture; □, kallikrein:amidase activity in the absence of kaolin; △, generation of kallikrein as determined by densitometry of the stained gels; □, per cent ³H-factor XII. *b*: ▲, generation of kallikrein in the complete reaction mixture plus RVV inhibitor II (114 µg), determined by densitometry of the stained gels; ■, per cent ³H-factor XII in the reaction mixture containing 23 µg of RVV inhibition II; ●, per cent ³H-factor XII in the reaction mixture containing 114 µg of RVV inhibitor II.

prekallikrein was converted to kallikrein (Fig. 2a, open circles), while 40% of the factor XII was still present as a single-chain molecule (Fig. 2a, open squares). After 5 min, 60% of the prekallikrein was converted to kallikrein, while only 30% of the factor XII was still in the single-chain form. After 5 min, the weight ratio of kallikrein-to-factor XII was nearly 40:1, and the conversion of factor XII to factor XII_a was occurring very rapidly. These data indicate that single-chain factor XII converts prekallikrein to kallikrein in the initial phase of the activation reaction in the presence of HMW kininogen and kaolin. This conclusion is further supported by the fact that the initial rate of prekallikrein activation by either factor XII or factor XII_a was nearly identical (Fig. 1a), and this would not be the case if the

prior conversion of factor XII to factor XII_a were required. These experiments also demonstrate that the latter part of the prekallikrein activation reaction is catalysed primarily by factor XII_a which is rapidly generated after the formation of kallikrein.

The activation of prekallikrein by single-chain ³H-factor XII was further examined in the presence of an excess of RVV inhibitor II, a low molecular weight serine protease inhibitor from Russell's viper venom. This protein inhibits bovine plasma kallikrein with a *K_i* of 0.29 nM (ref. 41) but has no effect on factor XII or factor XII_a. Thus, it is useful to restrict the effect of kallikrein on the activation of factor XII. The conversion of prekallikrein to kallikrein in the presence of a 5-M and a 25-M excess of the RVV inhibitor II relative to prekallikrein is shown in Fig. 2b. The weight ratio of ³H-factor XII-to-prekallikrein was 1:65. The formation of kallikrein was measured by SDS-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol as described for Fig. 2a. In these experiments, the rate of generation of kallikrein (Fig. 2b, solid triangles) in the presence of either a 5-M or a 25-M excess of kallikrein inhibitor was the same as that in the absence of the kallikrein inhibitor (Fig. 2a, open triangles). As before, no lag phase was observed. The conversion of factor XII to factor XII_a, however, was substantially reduced in the presence of the kallikrein inhibitor. With a 5-M excess of RVV inhibitor II, 35% of the prekallikrein was converted to kallikrein in 2.5 min, while 55% of the factor XII was in the single-chain form (Fig. 2b, solid squares). With a 25-M excess of RVV inhibitor II, 35% of the prekallikrein was converted to kallikrein in 2.5 min, while 70% of the factor XII was still in the single-chain form (Fig. 2b, solid circles). If the conversion of factor XII to factor XII_a were an absolute requirement for this reaction, then a lag phase would have been readily detected in these experiments and the initial rate would have been much slower than that found for an equivalent amount of factor XII_a. These data strongly suggest that single-chain factor XII converts prekallikrein to kallikrein in the presence of HMW kininogen and kaolin and does so at a very rapid rate.

Activation of factor XI by factors XII or XII_a

Similar experiments were then carried out for the activation of bovine factor XI by factor XII or factor XII_a in the same reaction conditions used in Fig. 1a, except that factor XI was substituted for prekallikrein. Factor XII readily activated factor XI in the presence of HMW kininogen and kaolin (Fig. 1b, solid circles). In these experiments, factor XI_a was measured by its esterase activity towards tosyl-L-arginine methyl ester. The initial rate of activation of factor XI by factor XII was 2–4 times slower than that of factor XII_a (Fig. 1b, solid triangles). The rate of activation of factor XI by factor XII_a, however, was almost the same as the activation of prekallikrein by factor XII_a or factor XII (Fig. 1a). As factor XII_a is a more effective catalyst than factor XII in the activation of factor XI, the generation of factor XII_a by kallikrein is required for the rapid activation of factor XI and the eventual formation of fibrin in whole plasma.

The cascade mechanism is initiated by factor XII

These data strongly suggest that the initial event in the activation of the intrinsic pathway of blood coagulation involves a substrate-induced catalysis by single-chain factor XII (Fig. 3). In this reaction, factor XII, bound to the kaolin surface, converts prekallikrein to kallikrein. The kallikrein thus generated converts the kaolin-bound factor XII to factor XII_a, which in turn converts factor XI to factor XI_a. Each of these three reactions is stimulated by HMW kininogen or kinin-free HMW kininogen. Human kinin-free HMW kininogen and bradykinin are formed after the generation of the kallikrein in the initial reaction. In the presence of calcium, factor XI_a then converts factor IX to factor IX_a. Eventually, thrombin is formed and this leads to the conversion of fibrinogen to fibrin¹. Although the present

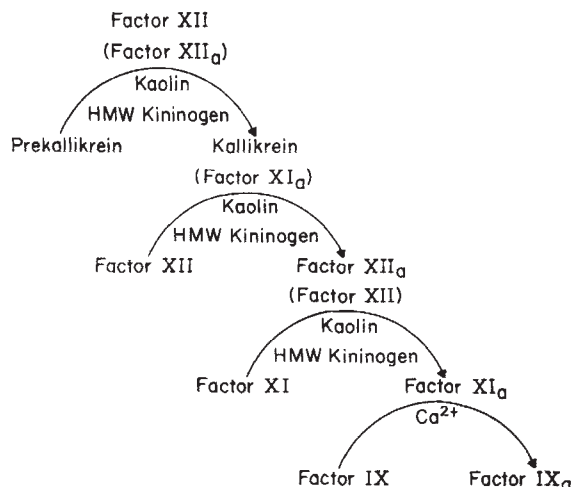


Fig. 3 A tentative mechanism for the surface activation of blood coagulation in the presence of kaolin.

experiments were carried out with clotting factors from bovine plasma, similar data have been obtained in preliminary experiments with purified clotting factors from human plasma. Thus, the mechanism shown in Fig. 3 seems applicable to other mammalian plasmas.

Factor XII_a will also activate prekallikrein after it is generated in the initial reactions. The activation of prekallikrein is not catalysed by kallikrein, factor XI_a or prekallikrein. Factor XI_a will activate factor XII when it is generated by factor XII_a (Fig. 3). We have not observed, however, an activation of human or bovine factor XII by factor XII_a. Recently, Wiggins and Cochrane⁴² have reported the cleavage of rabbit factor XII by factor XII_a. In whole plasma, the activation of factor XII is probably catalysed primarily by kallikrein rather than factor XI_a, as kallikrein is generated first and it is about four times more active in the activation reaction than factor XI_a (ref. 26). Thus, factor XI_a probably has only a minor role in the activation of factor XII. Factor XII will also activate factor XI in the presence of kaolin and HMW kininogen, although the rate is slower than that catalysed by factor XII_a. Thus, the initiation of blood coagulation by kaolin occurs at a faster rate in the presence of prekallikrein which is converted to kallikrein, and this in turn leads to the formation of factor XII_a. When plasma deficient in prekallikrein is preincubated with kaolin for approximately 10 min before the addition of calcium, it has a normal clotting time²². In these conditions, optimal levels of factor XI_a are probably generated by single-chain factor XII and the formation of factor XII_a via kallikrein is not required³². The correction of the kaolin clotting time of prekallikrein-deficient plasma by factor XII (refs 17, 27) can also be explained by the fact that elevation of the level of this protein will lead to the direct activation of factor XI and bypass the requirement for prekallikrein in these conditions.

Consequences of our studies

The present data provide a mechanism for the initiation of blood coagulation, as well as fibrinolysis, and kinin generation by single-chain factor XII in the presence of a surface, such as kaolin. By this mechanism, a trace of factor XII_a, kallikrein or some other plasma or cellular protease as previously suggested^{3,21}, is not required to initiate the reactions. Also, it is not necessary to postulate the participation of factor XII or prekallikrein as weakly active zymogens as suggested by Griffin and Cochrane³⁴, as factor XII is as active as factor XII_a in the activation of prekallikrein in the presence of kaolin and HMW kininogen. Whether or not this mechanism will be applicable for other negatively charged surfaces is not clear. The physiological importance of the surface activation reaction is evident when

blood comes in contact with a foreign surface *in vivo*. This occurs following the implantation of artificial heart valves where the incidence of thrombosis on the valve is 20–25% (ref. 43). Similar problems are encountered when patients with kidney failure are subjected to haemodialysis, when fibrin formation often occurs in the shunt.

Received 17 December 1979; accepted 7 May 1980.

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Distinctive sequence of human mitochondrial ribosomal RNA genes

I. C. Eperon, S. Anderson & D. P. Nierlich*

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

The nucleotide sequence spanning the ribosomal RNA (rRNA) genes of cloned human mitochondrial DNA reveals an extremely compact genome organization wherein the putative tRNA genes are probably 'butt-jointed' around the two rRNA genes. The sequences of the rRNA genes are significantly homologous in some regions to eukaryotic and prokaryotic sequences, but distinctive; the tRNA genes also have unusual nucleotide sequences. It seems that human mitochondria did not originate from recognizable relatives of present day organisms.

HUMAN mitochondrial DNA comprises a duplex closed circle of molecular weight 10×10^6 , which has been shown to code for several proteins¹ and RNA species². Two RNAs (12S and 16S) are conspicuous by their stability, methylation and equimolar synthesis^{3,4}, being the RNA components in 60S mitochondrial ribosomes^{5,6}. These unique genes⁷ are adjacent⁸, and were mapped relative to the 4S RNA genes⁹ and the *HpaII* cleavage points in mitochondrial (mt) DNA^{2,10}.

It has been suggested that mitochondria are derived by endosymbiosis from prokaryotes, based on the sensitivity of ribosomes to antibiotics¹¹ and initiation of polypeptides with formylmethionine¹². However, mammalian mitochondrial ribosomes and rRNAs are unusually small, and all but plant mitochondria also lack a 5S rRNA¹³. The rRNA sequences, being the only identified large genes with nuclear and prokaryotic counterparts, should clarify phylogenetic relatedness (for example, in chloroplasts the 16S rRNA gene¹⁴ has shown near identity to that from *Escherichia coli*). This comparison should also clarify the sequences necessary for the function of these small rRNAs.

Most of the *MboI* fragments from human placental mt DNA have been mapped and cloned in the *BamHI* site of the plasmid

pBR322 (ref. 15); these have been used to determine the rRNA gene sequences presented here.

DNA sequence analysis

Figure 1 shows the map established for the restriction enzymes *MboI* (ref. 11) and *HpaII* (ref. 10). The cloned *MboI* fragments 7, 14, 12, 3a, 18, 19 and 9 were sequenced using the dideoxy chain-termination method¹⁶. Single-stranded template was prepared by cleavage of the pBR322 moiety of the recombinant plasmid at the single sites for the enzymes *HindIII* or *Sall*, followed by exonuclease III digestion¹⁷ which results in degradation of each strand from the 3' end, exposing different strands in each case. An example is given in Fig. 2, which shows the sequence of the tRNA^{val} gene and the 3' end of the 12S rRNA gene.

Small inserts were sequenced using adjacent pBR322 restriction fragments as primers; larger inserts required the use of restriction fragments purified from the insert, which were often pretreated with exonuclease III to give sequence information within the region complementary to the restriction fragment¹⁸. Where the internal fragments were large, ribosubstitution of the 3' end of the fragment was used to generate a new 5' end for the extended chains on cleavage with alkali or ribonuclease. The published technique¹⁹ was modified to allow direct ribosubstitution of the fragments before addition of the template:

* Permanent address: Department of Microbiology, College of Letters and Science, University of California, Los Angeles, California 90024.



Fig. 1 The *MboI* restriction enzyme map of human mtDNA¹¹ is correlated with that of *HpaII*¹⁰, based on their alignments with some of the other enzyme cleavage sites marked and corrected according to the sequence in the region published here, which is shaded. The rRNAs are marked, as are the L-strand (■) and H-strand (●) sequence 4S rRNAs¹⁰. The origin and direction of H-strand synthesis are shown.

incubation of the fragment with a ribonucleotide triphosphate and the Klenow fragment of DNA polymerase I at 37°C allowed exchange of the 3'-terminal deoxyribonucleotide or 'filling-in' of the restriction fragment with the ribonucleotide. This eliminated the possibility that the overlapping ends of the exonuclease-degraded template could be substituted. EDTA was used to chelate Mn^{2+} during the reaction to prevent ribonucleotide incorporation during extension with deoxy- and dideoxynucleotides without recourse to column separation steps (I.C.E., unpublished).

Single-stranded template DNA prepared by exonuclease digestion can give a relatively high background and two other techniques were also used. One involved the separation of complete plasmid strands on alkaline RPC-5 columns (ref. 20 and B.A. Roe, manuscript in preparation) after cleavage of the closed circular duplex at a unique restriction enzyme site. This was most satisfactory where the pBR322 vector contained large inserts that were asymmetric with respect to the base compositions of the two strands, although recovery was variable. The second technique involved recloning of the isolated insert into the *BamHI* site of the double-stranded form (RFI) of a derivative of the single-stranded bacteriophage M13 mp2 (refs 21–23).

To establish the orientation of the *MboI* fragments, and to ascertain whether very small fragments might have been missed in the mapping experiments, fragments prepared from the pBR322 clones were used for priming on HeLa mt DNA that had been digested by exonuclease III from the *BamHI*, *EcoRI* or *XbaI* sites. The sequences obtained thus overlapped the *MboI* sites. Further confirmation was provided by sequence analysis of *AluI* fragments (A. R. Coulson and F. Sanger, personal communication). The sequence obtained is shown in Fig. 3, with the *MboI* sites and extent of the probable genes indicated.

Organization of the genome

The tRNA genes have been assigned to the sequences shown (Figs 3, 4) by detection of likely clover-leaf structures (using the computer program TRNASU: R. Staden, unpublished) and features of the sequence that are common to all previously identified tRNAs^{24,25}. The assignments correlate well with the

map positions identified by electron microscopy of HeLa 4S RNA-mt DNA hybrids. No further likely tRNA genes were found when the possibility of insertions in the anticodon loop, analogous to those found elsewhere^{26–28}, was allowed.

The rRNAs were exactly located by the accurate homology with short sequences from the 5' ends of the HeLa rRNAs², and found to about tRNA genes at these termini (Fig. 3). The 3' ends cannot be identified at present, although the extensive sequence homology to known small subunit rRNA sequences to within approximately 12 nucleotides from their 3' termini (Figs 5, 6a) suggests that the 12S 3' terminus is very close to the tRNA^{Val} gene. This proximity agrees with mapping by electron microscopy, which has shown that the gap between the two rRNA genes is less than 500 base pairs⁸ and that a 4S RNA maps between the two⁹. This closeness may involve a conjunction of the 12S rRNA and tRNA^{Val} genes as at the 5' end of both rRNA genes.

The lack of an intergenic sequence at some junctions suggests that a large initial transcript will be produced: this would agree with the large sizes of the HeLa mitochondrial transcripts hybridizing to the heavy (H) strand, the largest of which (9.0×10^5 MW, equal to the sum of the rRNAs) is found polyadenylated and unadenylated^{2,29}. The polyadenylated species has a short half-life compared with some other discrete mitochondrial 'poly(A)-containing' RNAs and is notable among these for being present in the polysomal fraction to a very small extent, as expected for a rRNA precursor. Resistance of restriction fragment-rRNA hybrids to exo- and endonucleolytic enzymes demonstrated that neither of the rRNA genes is 'split' by an intervening sequence in HeLa mitochondria².

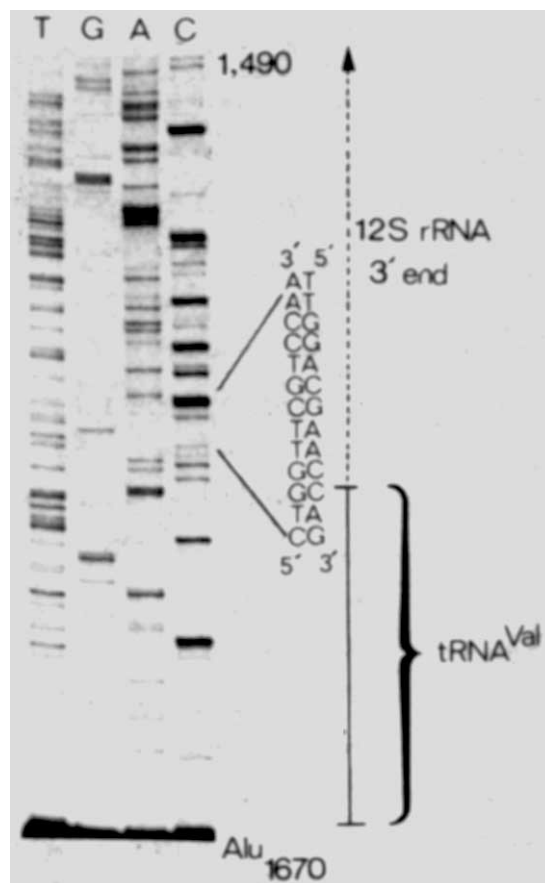


Fig. 2 A chain-termination sequencing gel showing the sequence through the tRNA^{Val} gene and the 3' end of the 12S rRNA, in the complementary sense. The primer is 223 bases long (the *AluI* fragment from 1,671–1,894 of Fig. 3), and the resultant sequence from priming on exonuclease-treated template (*AvaI* digest of *MboI* fragment 3a cloned in pBR322) can be read from this 6% thin gel³⁶, run for 4 h, to a total polynucleotide length of 400 bases, that is, to 1,490 on Fig. 3. The sequence through the junction of the two genes is shown.

In *E. coli*, processing of the 16S rRNA probably involves initial cleavage by RNase III of a large inverted repeat that flanks the 16S gene³⁰, followed by at least RNase M16 cleavage to generate the mature 5' terminus³¹. Eukaryotic nuclear rRNA transcripts also include large flanking sequences that are later removed^{32,33}, although the available sequence data do not suggest that these are inverted repeats (ref. 34 and K. G. Skryabin, personal communication). The 'butt-jointing' of mt rRNA genes at one or both end of the rRNAs implies a totally different processing mechanism, involving recognition of the

structure or sequence of regions within mature transcripts and simple cleavages around the rRNA genes if both mature from the same transcript.

This mechanism implies a novel method for the production of the 3' terminus of mature tRNAs, which in *E. coli* and *Bombyx mori* probably involves 3',5' exonuclease activity³⁵, as mitochondrial tRNA^{Phe} and tRNA^{Val} about rRNA genes at their 3' termini. However, single cleavage at the 5' terminus of the tRNA genes would not be unusual: such a cleavage is catalysed in *E. coli* by RNase P, the rate being dependent on the secondary

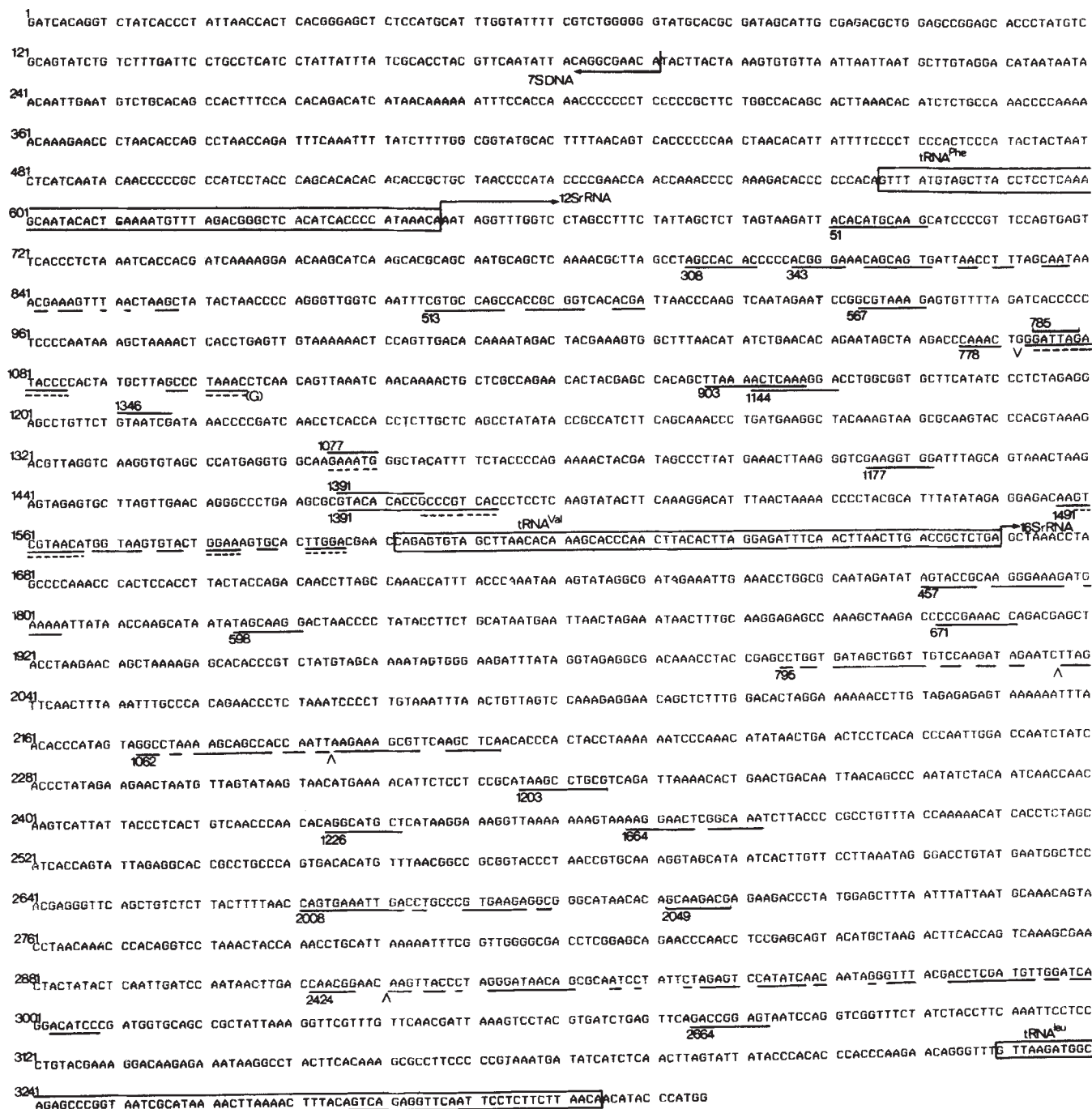


Fig. 3 The sequence of the region shaded in Fig. 1 is shown, with the tRNA genes boxed and the 5' termini of the 12S and 16S rRNA genes shown; this is L-strand sequence, which corresponds to the RNA sequence. Numbers down the left-hand edge are those of this mt sequence, and those adjacent to underlining are those of the *E. coli* rRNAs. The 5' terminus of 7S DNA, which is complementary to the strand shown, is also marked. Underlining shows regions of homology with the *E. coli* rRNAs ≥ 8 nucleotides, with surrounding smaller blocks in the same phase. A and V indicate that the *E. coli* rRNAs have one nucleotide more or less, respectively, in the areas of parallel sequence. Overlined regions are homologous to sequences ($\geq$ pentamers) identified by Woese *et al.*⁷² as universal among prokaryotes and kethoxal modifiable in 30S ribosomal subunits of *E. coli*. Broken underlining emphasizes those sequences that are identifiably homologous (assuming that sites would be in the same 5'-3' order along the two rRNA sequences) to sites identified by Herr *et al.*⁷³ as those in 16S rRNA of *E. coli* that must be unmodified by kethoxal for 30S-50S subunit association.

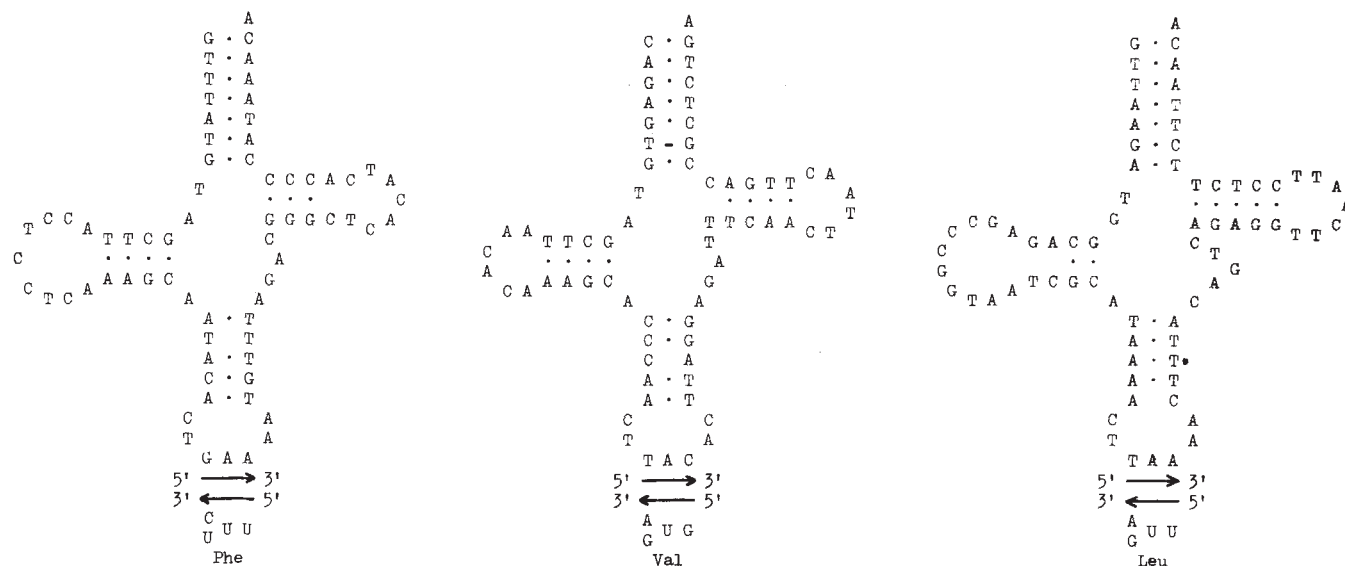


Fig. 4 A clover-leaf arrangement of the tRNA genes. The 3' CCA sequence common to all other tRNAs is not encoded. G-U wobble is assumed in the codons recognized⁴⁶. Loop I is the left-hand loop of this arrangement and often contains dihydrouridine, and loop IV is the right-hand loop which is usually characterized by the sequence G-T-pseudouridine-C.

or tertiary structure of the tRNA moiety of the substrate³⁶, and a similar *in vitro* activity has been isolated from the eukaryote, *B. mori*³⁵. Cleavage around both termini of mt tRNA sequences in the primary transcripts by an analogous activity to RNase P is a possible general processing mechanism in human mitochondria, agreeing with the approximate correlation found between the length of mapped inter-tRNA sequences and the sizes of encoded proteins² and with the generally larger sizes of the polyadenylated RNA species.

The origin of H-strand replication of HeLa mt DNA was initially mapped by locating the D-loop within large restriction fragments by electron microscopy³⁷, and later by hybridization of the D-loop-forming 7S single-stranded DNA fragment to small restriction fragments with subsequent primed synthesis³⁸. A sequence of the HeLa 7S DNA at its 5' end and adjacent restriction fragment sequences have been published³⁹. The placental sequence differs slightly from the HeLa sequence in that nucleotides 263–264 are A–C rather than just G, and there are three fewer Cs in the block 303–315.

The function of the region between the 5' end of the 7S DNA (H-strand sequence) and the 5' end of the first tRNA^{Phe} (light-strand sequence) seems likely to include that of initiating transcription of L-strand sense RNA, but there are no sequences resembling the initiation sequences found in *E. coli*⁴⁰ or those before the 5' end of transcripts in eukaryotes. Further, there are no obvious repeated sequences analogous to the two or more promoters^{41,42} or RNA polymerase binding sites⁴³ found before rRNA genes in *E. coli* and *Xenopus*⁴⁴ that have been postulated to play a part in the high level of transcription of these genes. The rRNAs in mitochondria are comparatively stable⁴ and this, rather than a high frequency of transcription, may account for their predominance *in vivo*.

Sequences of tRNA genes

With the exception of those flanking the cytochrome oxidase subunit II gene in human mitochondria⁴⁵, previously published mt tRNA sequences have come from *Neurospora* or *Saccharomyces* and have shown most of the features common to prokaryotic and eukaryotic tRNAs. Only the initiator tRNA from *Neurospora crassa*⁴⁶, for example, seems to have radically altered those nucleotides identified [from X-ray crystallographic studies on yeast cytoplasmic tRNA^{Phe} (ref. 47)] as being involved in the loop I–loop IV tertiary interactions.

In contrast, the sequences presented here (Fig. 4, drawn on the assumption that stem lengths are conserved) differ considerably from other tRNA sequences. This is most notice-

able in the lengths and sequences of loops I and IV, where many 'invariant' features are lacking, and in the poor base pairing in the stem to loop IV of tRNA^{Phe}. These changes may reflect altered possibilities of interaction between the two loops. The significance of the retained 'invariant' and common features cannot be assessed from so few sequences and will be discussed elsewhere.

Sequence analyses of the genes coding for the yeast ATPase subunit 9 (refs 48, 49) and cytochrome oxidase subunit II^{45,50} (in human and yeast mitochondria) have recently shown, by comparison with the yeast and beef protein sequences respectively, that threonine (in yeast only) and tryptophan (and perhaps methionine in human mitochondria) are coded for by triplets which otherwise have different specificities in the 'universal' genetic code. The codons corresponding to the tRNAs published here, UUC(U), GUA(G) and UUA(G), do not seem to be anomalous in protein–DNA sequence comparison, and so probably do code for the amino acids shown (Fig. 4).

Like the sequences of yeast and *Neurospora* mt tRNAs⁵¹, the human mt tRNA sequences published here show no greater homology to prokaryotes than to eukaryotes, seeming to form a rather distinct class. At some positions in tRNA sequences that are apparently characteristic of prokaryotes or eukaryotes, these genes frequently differ from both. The lack of agreement with a recent prokaryotic origin is emphasized by the inability of *E. coli* aminoacyl tRNA synthetases to charge mt tRNAs^{52,53}. The HeLa cytoplasmic enzymes were partially successful in some cases⁵³ with HeLa mt tRNAs, one of which involved valine incorporation despite the fact that the human placental mt tRNA^{Val} has lost the conserved positions found in prokaryotic, eukaryotic and yeast mt tRNAs.

The 16S ribosomal RNA gene

Because relatively little is known about mitochondrial ribosomes, the significance of the sequence shown in Fig. 3 must be brought out by comparison with known sequences.

The homology of *E. coli* 23S rRNA⁵⁴ to this 16S rRNA does not extend to the 3' end of the former sequence, resulting in some uncertainty about the length of the latter gene. Despite this and the considerable difference in probable lengths (2,904 to 1,559 nucleotides), there are regions of homology between the two. Regions with eight common nucleotides, with surrounding smaller blocks of homology in the same phase, have been shown in Fig. 3 where these regions occur in the same order in both sequences. The largest region of homology is found between nucleotides 2,910 and 3,010 of Fig. 3, corresponding to

nucleotides 2,422–2,522 of the 23S rRNA. This is within the region that has been identified as involved in the interface between *E. coli* ribosomal subunits, the RNA being protected against kethoxal modification on 30S–50S association⁵⁵, although the exact sites of modification are not conserved. More extensive homology to this region of the 23S rRNA has been reported in a partial sequence of the yeast mt 21S rRNA gene⁵⁴. Nucleotides 2,934 and 2,990 of Fig. 3 correspond to 21S rRNA nucleotides altered in chloramphenicol-resistant mutants of yeast and are therefore probably involved in binding the 3' terminus of aminoacyl tRNAs. Only 2 of the 16 kethoxal-modifiable sites found in *E. coli* 50S subunits⁵⁴ occur within the underlined regions, suggesting a low level of conservation of exposed sequences in general.

The 12S ribosomal RNA gene

By contrast with the absence of sequences from cytoplasmic large rRNAs, some of the yeast small (18S) cytoplasmic rRNA sequence^{6,7} is known, as is that of the *E. coli* 16S rRNA^{56,57}. The involvement of the 3' end of 16S rRNA in initiation of protein synthesis has led to the determination of short sequences at the 3' ends of many small subunit rRNAs (Fig. 5).

The degree of complementarity⁵⁸ and binding⁵⁹ of the 3'-terminal sequence of *E. coli* 16S rRNA to a polypurine stretch before the initiation codons in bacteriophage RNA has been correlated with the level of translation of these RNAs. Complementarity alone, however, does not fully explain the level of binding, or the translational preferences of ribosomes from various bacterial species^{60,61}. In contrast, eukaryotic ribosomes show comparatively little messenger discrimination; the 3'-terminal sequences of eukaryotic 18S rRNAs are highly conserved⁶², and no region of complementarity has been identified in eukaryotic mRNAs that has any constant relationship to the initiation codon. The eukaryotic ribosome-Met tRNA^{Met} complex⁶³ may therefore not recognize internal regions of mRNA, but could 'scan' the mRNA for the first initiation codon⁶⁴, after binding near the 5' end^{65,66}; complex binding⁶⁵ and the monocistronic translation of prokaryotic mRNA are enhanced by a 'cap' (refs 67, 68).

The comparison shown in Fig. 6a clearly illustrates that the small subunit rRNA from human mitochondria lacks the poly-

purine-complementary sequence (CCUCC) found in prokaryotes. Although the exact 3' end of the 12S rRNA has not been established, it is apparent (Fig. 6b) that any extended stem would be less stable than that of the analogous structure in *E. coli* if the rRNA gene does not overlap the tRNA^{Val} gene. This stability may be necessary for dissociation (after initiation) of the prokaryotic CCUCC sequence from the mRNA⁵⁹, and hence may be diagnostic of such a mode of initiation. No region of complementarity to the 3' end of 12S rRNA has been found appropriately near the initiation codon for the cytochrome oxidase subunit II⁴⁶ or other structural genes (B.G. Barrell, personal communication).

The absence of these prokaryotic characteristics suggests that there is no sequence-specific binding of ribosomes and internal initiation, but rather that the long mitochondrial transcripts are processed to monocistronic units as in eukaryotes. If so, initiation seems likely to be dependent on the 5' terminus *per se* or the first AUG codon for transcripts are not capped and in at least one case there would be no leader sequence (the AUG codon abuts a tRNA gene) unless translation preceded processing⁴⁶. No eukaryotic transcripts are known where there is no leader sequence, although the cap structure does not seem to be invariably necessary for efficient translation, and the single prokaryotic example (λ prn transcript⁶⁹) is translated inefficiently. It is interesting, however, that the λ prn transcript is translated; this suggests that initiation will occur at the AUG nearest to the 5' end of mRNA, or that the 5' end of mRNA is bound by the 30S-initiation factor complex. Mitochondria may represent a situation where there is no 12S-mRNA complementarity to favour internal initiation, nor a cap to enhance 5'-terminal binding, but a simpler mechanism underlying those developed elsewhere. If this is the case, the persistent failure of non-mitochondrial *in vitro* or *in vivo* systems to produce other than at best fragmentary translation products from mt RNA or DNA^{70,71} may be ascribed to the use of the codon TGA (normally a terminator) to code for tryptophan^{46,51}, rather than a lack of initiation. One would, however, expect discrete products representing N-terminal peptides of proteins.

The 12S rRNA shows several other regions of homology to both *E. coli* and yeast small subunit rRNAs (Figs 3, 5, 6). The homology around the stem-loop structure near the 3' end is

mt 12S	1385	CTACGATAGC	CCTTATGAAA	CTTAAGGTC	GAAGGTGGAT	TTAGCAGTAA	ACTAAGAGTA
<i>E. coli</i> 16S	1307	TGGAGTCTGC	AACTCGACTC	CATGAAGTCG	CAATCGCTAG	TAATCGTGA	TCAGAAATGCC
Yeast 18S		AGAGCATCT	AATATCTCT	CTTCAACGAG	GAATTCCTAG	TAAGCGCAAG	TCATCAGCTT
mt 12S	1385	CTACGATAGC	CCTTATGAAA	CTTAAGGTC	GAAGGTGGAT	TTAGCAGTAA	ACTAAGAGTA
mt 12S	1445	GAGTGCTTAG	TTGAACAGGG	CCCTGAAGCG	CGTACACACC	GCCCGTCACC	-----
<i>E. coli</i> 16S	1367	AGGCTGAATA	CGTTCCGCGG	CG-----T	TGTACACACC	GCCCGTCACA	-----
Yeast 18S		GCGTTGATTA	CGTCCGTGCC	CT-----T	TGTACACACC	GCCCGTCGCT	AGTACCCATT
mt 12S	1445	GAGTGCTTAG	TTGAACAGGG	CCCTGAAGCG	CGTACACACC	GCCCGTCACC	-----
mt 12S		-----	-----	-----	-----CTCCT	CAAGTATACT	TCAAAGGACA
<i>E. coli</i> 16S		-----	-----CCAAT	GGGAGTGGGT	TGCAAAAGAA	GTAGGTAGCT	TAACCTTCGG
Yeast 18S		GAATGGCTTA	GTGAGGCGCT	AGGATCTGCT	TAGAGAAGGC	GCACAACTCCA	TCTCAGAGCG
mt 12S		-----	-----	-----	-----CTCCT	CAAGTATACT	TCAAAGGACA
mt 12S	1520	TTTAACTAAA	ACCCCTACGC	ATTTATATAG	AGGAGACAAG	TCGTAACATG	GTAAGCTTAC
<i>E. coli</i> 16S	1454	GAGCGCGCTT	ACCACTTTGT	GATTCATGAC	TGGGGTGAAG	TCGTAACAAAG	GTAACCTTAC
Yeast 18S		GAGAATTGGG	ACAAAOTTTG	TCATTTCGAG	GAACATAAAG	TCGTAACAAAG	GTTCCTGAG
mt 12S	1520	TTTAACTAAA	ACCCCTACGC	ATTTATATAG	AGGAGACAAG	TCGTAACATG	GTAAGCTTAC
mt 12S	1580	TGGAAAGTGC	ACTTGGACGA	ACCAGAGTGC			
<i>E. coli</i> 16S	1514	GGGAACCTGC	GGTGGG-TC	ACCTCCTTA	OH		
Yeast 18S		GTCACACCTGC	GGAAGGA-TC	A-----TTA	OH		
mt 12S	1580	TGGAAAGTGC	ACTTGGACGA	ACCAGAGTGC			

Fig. 5 The 3' termini of mitochondrial *E. coli*^{56,57} and yeast³⁴ small subunit rRNAs have been aligned to show the major blocks of homology, introducing gaps where appropriate. The scores for homologous nucleotides out of the total possible in this alignment are 45%, 57% and 45% for the pairs mitochondria: *E. coli*, *E. coli*: yeast and yeast: mitochondria, respectively. The mt DNA sequence has been extended for seven nucleotides into the tRNA^{Val} gene, illustrating the lack of homology there.

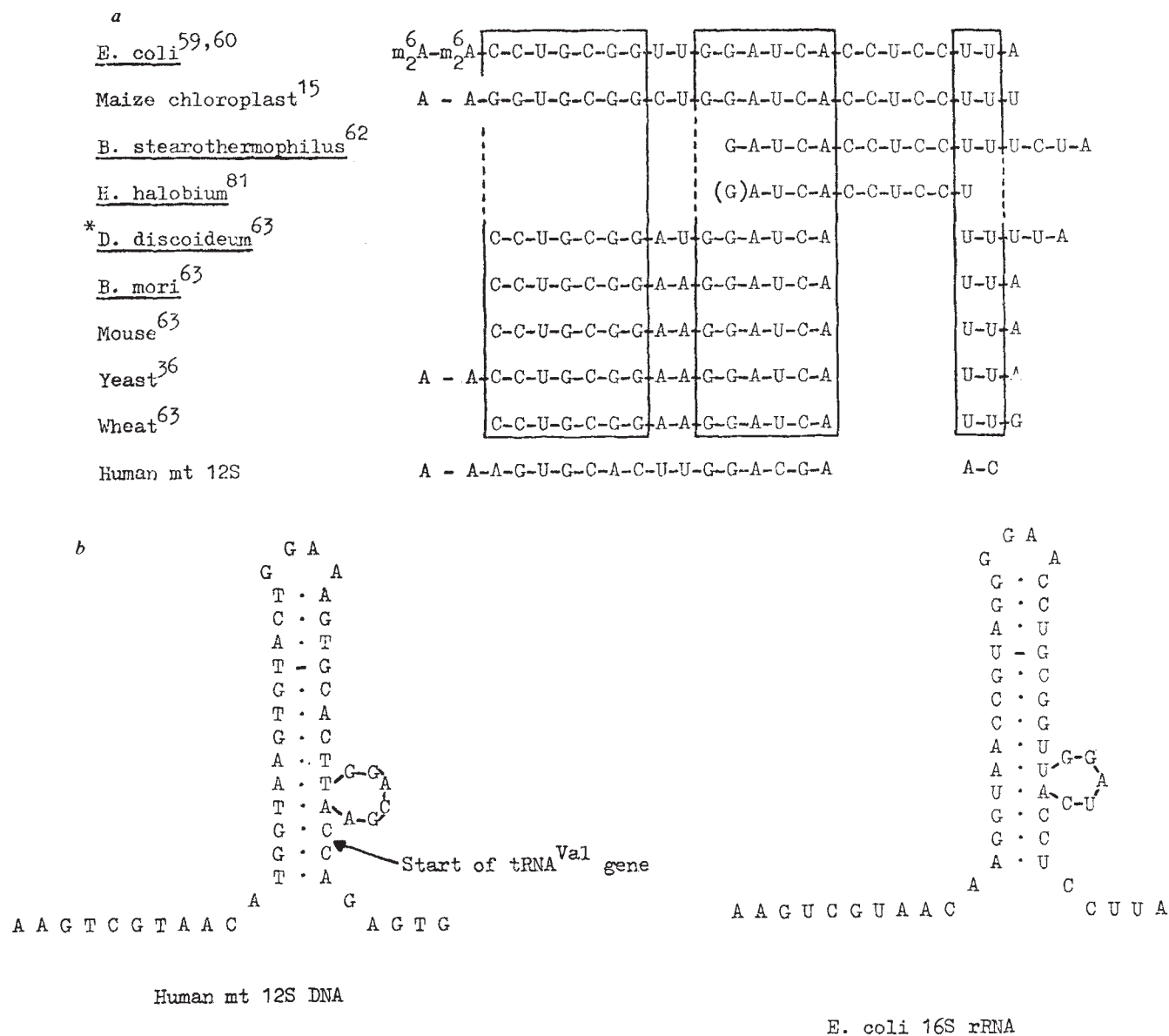


Fig. 6 **a**, The 3'-terminal sequences of small subunit rRNAs. The solid lines enclose regions of absolute homology in all previously determined sequences, including some prokaryotes not included here. Broken lines indicate incomplete sequences or a short 3' terminus. **Dictyostelium discoideum*. **b**, The small subsidiary loop proposed for *E. coli*⁵⁹ and the extended stem (of sequence GGAUCACCU) are unlikely to form in mitochondria unless the 12S gene overlaps the tRNA^{Val} gene.

broken, but differences from the other sequences are such as to retain the possibility of base pairing. The TG base pair and its flanking pairs are found in all the sequences known. The large block of homology in the region of nucleotides 1,476-1,495 (1,391-1,408 in the *E. coli* sequence, Fig. 5) is strongly conserved in mitochondria, eukaryotes and prokaryotes. Like the region just before the 3' stem-loop, there are important functional aspects to this sequence in *E. coli*, for part is a sequence that is universal among prokaryotes and accessible to modification by kethoxal in 30S subunits⁷², and the remainder has been identified as crucial for 30S-50S subunit association⁷³. Several other sequences are homologous to those found to be universal and accessible to modification in prokaryotes (Fig. 3), and 7 of the 14 kethoxal-modifiable sequences in *E. coli* 16S rRNA that are implicated in subunit association are recognizable in the 12S sequence. One of these is very similar to one of two sequences that might be involved in base pairing to 23S rRNA in *E. coli*^{55,73}, but there are no complementary sequences (>6 base pairs) in the mt 16S rRNA. The latter indeed has no regions absolutely homologous to the few association-protected oligonucleotides identified in *E. coli* 23S rRNA⁵⁵.

Evolution of human mt DNA

Although only one other small rRNA gene (16S rRNA of *E. coli*) has been sequenced, the sequences of the many small oligonucleotides produced by exhaustive ribonuclease T1 digestion of 16S rRNA have been used to deduce relatedness and highly conserved sequences among prokaryotes. Apart from obvious blocks of homology, comparisons of extensive rRNA sequences and judgements of similarity are hampered by the fact that the functional importance of the structure is not known in most regions. Unlike some proteins, it is not possible to draw conclusions as to similar chemical nature, structural role or frequency of substitution by the comparison of non-identical sequences. The use of catalogues of oligonucleotides, each of which represents a phylogenetic character and is scored as absent or present, is therefore attractive, and does not require attempts at alignment of different sequences.

Previous evidence for an endosymbiotic origin of mitochondria has sought to demonstrate recent common ancestry with contemporary prokaryotic forms. However, these arguments have generally relied on features that are not due to mitochondrially coded components⁷⁴⁻⁷⁶, and often the similarities are

Table 1 Degree of homology in small subunit rRNAs

Sequence	Oligonucleotide catalogue				
	<i>E. coli</i>	<i>R. spheroides</i>	<i>B. stearo-thermophilus</i>	<i>H. halobium</i>	<i>Wheat</i> mt 18S*
Human mt 12S rRNA gene	15(9.5)	34(11)	22-28‡(9)	30(12)	20(17)
<i>E. coli</i> 16S rRNA	†	167(17)	151(13)	53(19)	87(28)

Values given are the total number of nucleotides in ribonuclease T₁-generated oligonucleotides (≥hexamers) that exactly match the two sequences, assuming a requirement for a G nucleotide before the oligonucleotide sequence. This stringency, together with the size requirements, reduces the number of *E. coli* oligonucleotides that match the 12S rRNA from 38 (see text) to 2 (a hexamer and a nonamer). The values in parentheses are the results expected when these catalogues are tested in this manner against randomly chosen sequences of the same length as the 12S or 16S rRNA (≈954 and 1,541 nucleotides, respectively). Oligonucleotides of unknown sequence were omitted.

* Wheat mt 18S and wheat cytosol 18S score 31, approximately the expected random level, when matched.

† As a test of the method, virtually all the oligonucleotides listed in ref. 72 matched the DNA sequence.

‡ Of the oligonucleotides which were shown with ambiguous sequences, one matched only when one of the two possible sequences was assumed.

more likely to have arisen by chance or convergence than are extensive sequence homologies.

The tRNA structures, unique genetic code and 3'-terminal sequence of the 12S rRNA suggest that mitochondria are less close to prokaryotes than the latter are to eukaryotes. This was further explored using oligonucleotide sequences from the published catalogues of small subunit rRNAs, which were fitted to the 12S rRNA gene sequence using the computer program SEQFIT⁷⁷. Of the 114 oligonucleotides produced from *E. coli* 16S rRNA⁷², only 38 matched the 12S rRNA, including 9 of the 29 'conserved' sequences but 14 of the 32 universal prokaryotic sequences⁷². With just 500 nucleotides known from the 5' and 3' termini of yeast cytoplasmic 18S rRNA (ref. 34 and K. G. Skryabin, personal communication), 29 *E. coli* oligonucleotides matched, 8 being conserved and 12 being universal sequences, suggesting (because the oligonucleotides do not all map at the ends of 16S rRNA) a far higher level of shared oligonucleotides (see Fig. 5) between prokaryotic and cytoplasmic rRNAs than either shares with mt rRNA.

To test evolutionary relatedness more stringently, hexanucleotides or larger from several catalogues were matched against the 12S mt rRNA and *E. coli* 16S sequences, and the total number of nucleotides in those oligonucleotides that matched was calculated (Table 1). These values are higher than those expected from matching unrelated sequences of the same lengths as those of the two rRNA genes to the individual catalogues. Table 1 shows that the Gram-negative bacterium *Rhodospseudomonas spheroides*⁷⁸ and the Gram-positive *Bacillus stearothermophilus*⁷⁹ are far more closely related to *E. coli*

than either is to human mitochondria, contrary to conclusions from comparisons of respiratory chains⁷⁵ and from matrix-of-differences trees based on cytochrome *c* and 5S RNA homologies⁷⁶. The close homology among all tested eubacterial rRNAs^{79,80} confirms the distinctiveness of the mt 12S rRNA. Despite the molecular peculiarities of the 'archaeobacterium' *Halobacter halobium*⁸¹, it seems to be more closely related to eubacteria than to human mitochondria. The approximately equal homology of eukaryotes and eubacteria with Archaeobacteria⁸⁰, together with the higher homology between the *E. coli* 16S rRNA and yeast cytoplasmic 18S rRNA (discussed above and shown in Fig. 5) than the homology of either with mt rRNA, suggests that the representatives tested from the major groups of organisms are more closely related to each other than to human mitochondria.

A catalogue from wheat mt rRNA has shown that, although not closely akin to prokaryotic sequences, it is nonetheless further removed from the wheat cytoplasmic rRNA⁸²; it can be added from Table 1 that human mt rRNA is at least as distant. 5S rRNAs have only been found in plant mitochondria; if they are absent in other mitochondria the sequences of the larger rRNAs may differ for this reason. A difference between mt rRNAs from different organisms was expected, yeast mitochondria differing in rRNA methylation^{83,84}, arrangement of the genome⁸⁵ and genetic code from mammalian mitochondria, but the implied distance from wheat is surprising. Further work on the difference between cytoplasmic and mitochondrial rRNA from different organisms will be required to show the relative extents of divergence of these sequences and thus the patterns of selection and drift in mitochondria.

From the results presented here human mitochondria seem to represent a radical departure from previously examined organisms. The compact genome organization is probably linked to a requirement for processing so as to free the tRNAs and rRNAs, and perhaps to allow 5'-end recognition of mRNAs. Although there are conserved features, and perhaps functions, of these rRNA sequences, the extent of the differences from other rRNA genes has consequences for the mechanism of initiation of protein synthesis and argues, at least, for a more ancient origin of any endosymbiosis than 'recent common ancestry' (ref. 76) with prokaryotes or eukaryotes.

We thank Dr F. Sanger for encouragement and criticism, Dr B. G. Barrell and Mr R. Staden for advice on the computer techniques used, Dr B. A. Roe for strand separation on RPC-5, and Dr G. Attardi for the gift of HeLa mt DNA and for communicating results before publication. S.A. is a Research Fellow of the Cystic Fibrosis Foundation of America and D.P.N. was supported by a grant from the USPHS.

Note added in proof: The sequence of the region between nucleotides 324 and 933 of Fig. 3 has been determined in HeLa mt DNA; the 5' sequence of the 12S rRNA differs only in being A at nucleotide 750 (ref. 87).

Received 21 January; accepted 19 May 1980.

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LETTERS

Discovery of a very distant supernova

M. R. S. Hawkins

Royal Observatory, Blackford Hill, Edinburgh EH9 3HJ, UK

The luminosity and rapid time variation of supernovae suggest that they could be powerful cosmological probes. The main obstacle to such use has been the difficulty of finding sufficient high-redshift examples in time to make the necessary observations of the light curve. Here a very distant ($z \approx 0.5$) supernova is reported together with photometric observations of its light curve.

On a given night a deep UK 1.2-m Schmidt plate would be expected to show between one and ten supernovae depending on the supernova rate and the usable limiting magnitude. In the present observing programme one sky-limited plate was taken per night, clear of Moon, for 3 weeks between two lunations. The plates could then be compared and a light curve measured for any supernova discovered with maximum near the beginning of the series. This could then be compared with a measured or estimated redshift. The main run of observations was made during August 1978 with the UK 1.2-m Schmidt in Australia. The field was centred on 21 h 28 min, -45° (1950) at a galactic latitude of -47° chosen as a compromise between time available on the telescope and minimum distance from the South Galactic Pole; the plate material is described in Table 1.

A Zeiss blink comparator was used to search for supernovae. Initially two plates were selected; the early epoch J3653, and J4410, a plate of similar quality near the beginning of the run. This produced 36 candidates which were appreciably brighter on the later plate. These images were then blinked with J4458, taken a month after J4410. Six of the candidates were found to have dimmed to their original magnitudes, and three of these

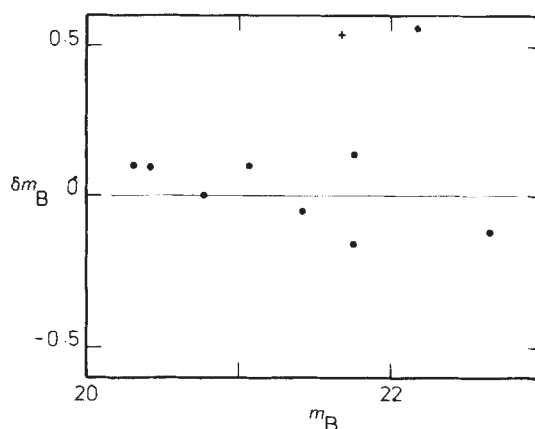


Fig. 1 Plot of residuals between mean magnitudes and magnitudes for plate J4394 against magnitude. +, The supernova image.

were too faint to measure photometrically. The remaining prime candidates—SN1, SN4 and SN6—were all ~ 2 mag above the plate limit at their brightest. SN4 and SN6 appeared to be stellar with no obviously associated galaxy, but SN1 was clearly resolved.

To obtain magnitudes three separate measurement procedures were used. A 'first look' was obtained by scanning in one dimension across the candidate image and four nearby comparison images spanning the full range of variation, using a Joyce-Loebl microdensitometer. The readings were converted to intensity using the step wedge calibration, and the area under the curves integrated to give relative magnitudes.

The magnitude scale was checked against photoelectric and electronographic standard magnitudes and no significant deviation from a slope of unity was found, even for brighter stars where saturation effects might be expected. Zero points were obtained by averaging the readings of each star and then plotting the measures for each plate against these mean magnitudes. A plot of relative magnitude of the candidate images against epoch revealed that SN4 and SN6 were not supernovae. They were both of more or less constant brightness on all plates except J4410, where they flared up by about one magnitude. Concentrating on SN1 an electronographic sequence was obtained of the field to fix the zero point of the mean magnitudes, which showed that at its brightest, the candidate image had $B = 21.4$ mag. The SN1 field was now remeasured on the Perkin-Elmer Data System (PDS) at the Royal Greenwich Observatory, including about eight comparison images comprising stars and galaxies, measured in square rasters. The images were analysed in two ways; first the volume under the images was integrated,

Table 1 UK 1.2-m Schmidt plates measured

Plate no.	Date UT	Exposure (min)	Seeing (arc s)
J3653	8.10.77	70	DL-1
J4381	25.7.78	45	2-3
J4394	27.7.78	70	DL
J4401	30.7.78	70	DL
J4410	31.7.78	70	1
J4416	3.8.78	65	4
J4425	4.8.78	75	4
J4436	9.8.78	70	3
J4458	23.8.78	70	1

Field centre 21 h 28 min $-45^\circ 00'$ (1950); emulsion IIIaJ; filter GG395. DL, diffraction limited.

and second a gaussian of variable height and radius was fitted to the image. These and the Joyce-Loebl measurements all gave substantially the same light curve, although they differed slightly over the measurement of individual images. The PDS integration method gave the smallest scatter and we use those measurements here.

Figure 1 shows residuals between plate J4394 and mean magnitude plotted against magnitude; the r.m.s. scatter of the residuals is 0.11 mag. The supernova candidate is shown by a cross, and the mean magnitude is taken from the early and late epoch plates; the cross is a 5σ point. Figure 2 shows magnitude plotted against epoch for SN1, the scatter being as expected, similar to that in Fig. 1. It is not usually possible to see the supernova as a distinct stellar image superimposed on an underlying galaxy on even the best plates. It looks more like a faint galaxy which suddenly becomes brighter and more star-like and then fades to its original form. The best early epoch plate (J3376) for SN1 shows a faint roughly circular galaxy with a rather compact centre. A positive classification of the galaxy type is not feasible for such a faint image but the general morphology is suggestive of an elliptical. J4381 is the first plate of the main series and here the image has become more point-like and distinctly brighter. On the next plate J4394, taken in very good seeing, the image is just resolved into a faint star-like object about 1 arc s west of the galaxy centroid. This is confirmed by positional measures from the PDS. For the other plates the image steadily fades as shown in Fig. 2, until it regains its early epoch magnitude. This object has all the properties expected of a distant supernova, and apart from some unlikely coincidences is hard to classify in any other way. Distinguishing a type I from a type II supernova is always difficult, even for nearby objects where spectroscopic information is available. Type II objects are, however, observed relatively infrequently, and if, as seems likely, the underlying galaxy is not a late-type spiral, a type II can be ruled out. To measure the decay time, the magnitudes in Fig. 2 must be modified by subtracting the flux from the underlying galaxy. The resulting light curve from the early and late epoch plates is shown in Fig. 3. A straight line was fitted to the readings to give the decay time, the best fit being

$$5.8 \pm 0.5 \text{ days mag}^{-1}$$

Repeating the fit after removing individual readings, and changing the value for the underlying galaxy by the standard error, still gave the best fit slope within the above error limits. Note that the last two observations, which after galaxy subtraction were the most uncertain, were not used. The resulting least-squares slope was $6.16 \pm 0.67 \text{ days mag}^{-1}$ which is not significantly different from the value given above. The r.m.s.

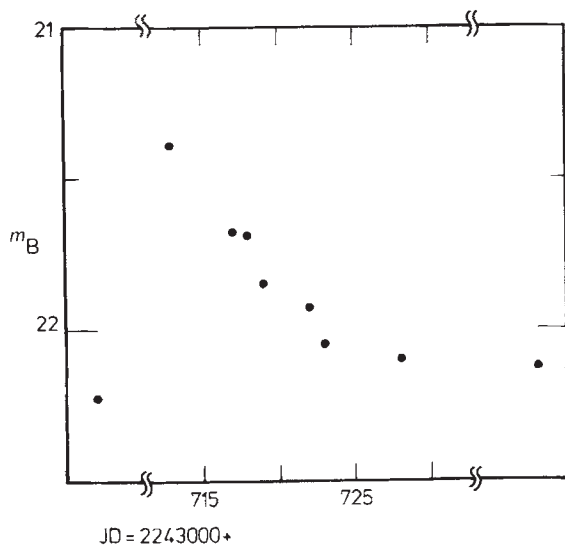


Fig. 2 Plot of magnitude against epoch for the supernova image.

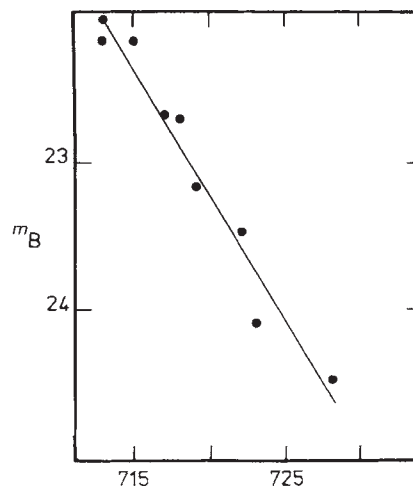


Fig. 3 Plot of magnitude against epoch for the supernova image after subtracting background galaxy flux. Also shown is the least-squares best fit straight line.

difference of the stars about the best fit line was 0.19 mag, larger than the error in Figs 1 and 2. This is to be expected due to the effect of subtracting the underlying galaxy.

Rust¹ has found that the local value for the decay rate of a type I nearby supernova is $8.32 \pm 1.95 \text{ days mag}^{-1}$ which is compatible with the result found above, although SN1 is clearly not a local object. Unfortunately, we cannot yet obtain a redshift for the underlying galaxy due to its faint magnitude of $B = 22.23$. However, the brightest magnitude in Fig. 3 is $B = 22.06$ which sets a lower limit to the supernova brightness at maximum. Rust¹ found a mean absolute magnitude at maximum of $m_B = -19.53$ with a dispersion of 0.94 mag. The mean value gives a redshift of $z \approx 0.6$ which in a Friedman cosmology would imply an increase of the decay time given by

$$T_0 = T_1(1+z) \quad (1)$$

where T_1 and T_0 are the intrinsic and observed decay rates respectively. Thus the expected decay rate at the redshift of 0.6 would be $13.3 \pm 3.1 \text{ days mag}^{-1}$, which puts the observed value of $5.8 \text{ days mag}^{-1}$ at 2.5σ , and in fact the slowest supernova known would have a decay rate of $7.2 \text{ days mag}^{-1}$ at this redshift. Even if the absolute magnitude of the supernova were 2σ fainter than the mean value at $m_B = 17.6$, a redshift $z \approx 0.3$ would be implied giving an expected decay rate of $10.8 \pm 2.5 \text{ days mag}^{-1}$; this is still 2σ above the observed value. The most natural interpretation of these observations is that light from the supernova has not undergone a Doppler shift.

Another important effect must be taken into consideration. De Vaucouleurs and Pence² have pointed out that the change in the supernova spectral energy distribution as a function of time alters the observed decay rate in a particular bandpass with redshift—the observed magnitudes must be modified by a time-dependent k -correction. This makes supernovae appear to decay more quickly at high redshifts, and de Vaucouleurs and Pence give as the correction factor

$$T_0 = T(1 + 1.08z) \quad (2)$$

where T is the observed decay time and T_0 is the decay time which would be observed were the passband redshifted to the rest frame of the supernova. The relation (2) is only claimed by de Vaucouleurs and Pence up to a redshift of $z = 0.15$; however, if this holds at the redshift of SN1 comparison of relations (1) and (2) shows that there will be essentially no observed change in the decay rate as a function of redshift in a Friedman cosmology, and that for a non-expanding universe the decay rate would actually become smaller. Applying the correction to the present observations puts the observed decay time within the bracket

represented by the Friedman cosmology, and is evidence that the Universe is expanding; rejection of the correction implies a non-expanding Doppler cosmological redshift.

I thank the UK 1.2-m Schmidt Unit, Miss Farooq Saedi, Dr B. D. Kelly and Dr R. S. Stobie for assistance.

Received 18 April; accepted 10 June 1980.

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A 2.2-Hz modulation of auroral electrons imposed at the geomagnetic equator

D. R. Lepine, D. A. Bryant & D. S. Hall

SRC Rutherford and Appleton Laboratories, Ditton Park, Slough SL3 9JX, UK

The direct observation of high frequency ~ 3 Hz modulation of electron intensities during pulsating aurora is comparatively recent although, from TV and photometric studies, modulation of the visual aurora at these frequencies has been recognized for over a decade. We report here 2.2 ± 0.5 Hz oscillations in the intensities of 4–25 keV electrons producing a pulsating aurora. The electrons were measured from a Petrel sounding rocket launched from Kiruna, Sweden on 25 January 1979. The oscillations like the slower 1–20 s pulsations, exhibit a marked velocity dispersion implying an equatorial origin for both forms of modulation. VLF—hiss observed at about the same time at the Satellite GEOS 2, operating in the same equatorial region, shows remarkable similarities to the modulation in the particle fluxes. A connection between VLF emissions and auroral pulsations has been suggested by Coriniti and Kennel who argue that low frequency, 5–300 s period, micropulsations modulate whistler-mode wave amplitudes leading to variations in the rate of pitch-angle scattering of electrons from the magnetosphere. The isotropic angular distributions reported here suggest that this mechanism acting alone cannot explain the modulation in the present case.

A Petrel rocket, designated P216 in the UK National Programme, was flown from Kiruna, Sweden, to compare electron intensities seen in pulsating aurora with related parameters at

the magnetically-conjugate geostationary satellite GEOS 2. The rocket was launched at 21.07.33 UT on 25 January 1979 during the recovery phase of a magnetic substorm. A low-light television, operating down range, showed that for part of its flight the rocket was in the vicinity of patches of aurora that pulsed irregularly with durations of maximum brightness of between 1 and 20 s. Electron intensities at the rocket, measured with channel multipliers and electrostatic analysers, exhibited similar pulsations. We are concerned here with shorter-period (0.46 ± 0.05 s) oscillations that occur in 4–25 keV electron intensities, at several intervals during the rocket flight superimposed on the maxima of the slower pulsations¹. Particle modulation in the 3 ± 1 Hz frequency range has been observed previously² at low energies (< 70 eV), and more recently at high energies³ (> 25 keV). The present observations are significant in that particles within the 4–25 keV range carry sufficient energy to account for the observed light emission and, therefore, may cause a 2–3 Hz modulation in light intensity frequently observed during pulsating aurora^{4,5}. During these particular oscillations, however, due to the combined effects of velocity dispersion and the narrow range of spectrum energies involved, the precipitated energy-flux variations amount to only $\pm 3\%$ of the ~ 25 mW m⁻² energy-flux at pulsation maximum. Because of the low auroral intensity (~ 2 kR at 427.8 nm), this level of modulation equals the noise level of the optical instrumentation and we cannot confirm that the particle modulation reported here leads to a modulation in the optical brightness.

Examples of the oscillations are contained in Fig. 1, an energy-time spectrogram of electron intensities for the flight-time interval 217–250 s. These intensities were measured at pitch-angles $20 \pm 3.5^\circ$ with a detector whose electrostatic analyser swept through the energy range 1.2–25 keV every 143 ms. In the interval shown the rocket altitude ranged from 170 to 180 km. Oscillations at 2.2 ± 0.2 Hz are strongest during intervals A and B in which it is very noticeable that intensity changes at higher energies precede the corresponding changes at lower energies. This effect can be readily explained as velocity dispersion due to a distant source of modulation. A dispersion in the times of occurrence of 1–20 s pulsations at different particle energies was discovered by Bryant *et al.*⁶, confirmed on subsequent occasions^{7,8}, and indeed found to be the case in this event¹. The present results are, we believe, the first indication of dispersion in shorter-period oscillations.

To test more closely the hypothesis of a distant source of modulation, we have examined the intensity changes recorded by other detectors operating continuously at energies 3.9, 7.7 and 11.5 keV. These constant-energy detectors were sampled every 0.5 ms as they scanned through the pitch-angle range 15 – 55° every 57 ms, all sampling the same pitch angle at the

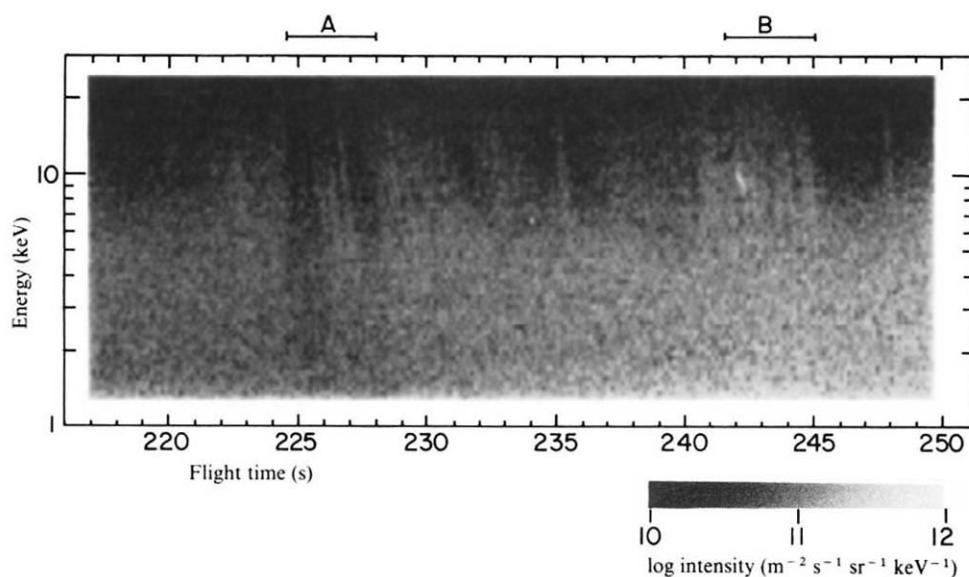


Fig. 1 Electron intensities measured at P216 during an interval of temporal changes. The intensities were measured at pitch angles $20 \pm 3.5^\circ$. 2.2 Hz oscillations occurring during the intervals A and B are discussed in the text.

same time. In the intervals A and B intensities were found to be isotropic over the observed pitch-angle range at all three energies. Figure 2 shows intensity variations at the three energies in terms of fluctuations about a mean intensity at that energy. It is immediately apparent that the oscillations are quasi-periodic with a mean frequency of 2.2 Hz, and that the amplitude and phase are energy dependent. The oscillations are, when the time shift is taken into account, well correlated.

Because the vertical separation in Fig. 2 is proportional to reciprocal of velocity, sloping lines linking the oscillations provide clear evidence of velocity dispersion, if we assume that modulation is simultaneous at all energies and that particles travel with constant speed after modulation. The data clearly support the dispersion hypothesis, and point to a source distance of $42 \pm 5 \times 10^6$ m. The length of a particle trajectory from a rocket altitude to the geomagnetic equator ranges from, in the dipole approximation, 41 to 43×10^6 m for the pitch angles (15 – 55°) sampled here, so the analysis performed in Fig. 2 therefore suggests that the source of modulation is located in the equatorial region at a radial distance of about $5.4 R_E$. The use of the dipole approximation requires justification, particularly when there is evidence for, on some occasions, a considerable stretching of field lines in the midnight sector of the magnetosphere. Using a comparison of plasma measurements made by the Vela satellites at $18 R_E$, with satellite photographs of the

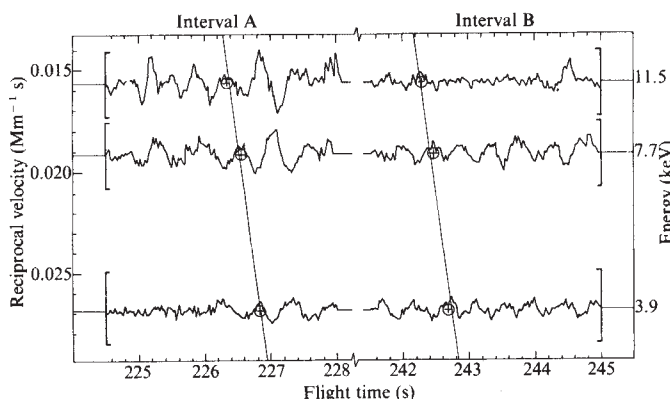


Fig. 2 Velocity dispersion of 2.2 Hz oscillations in the intervals A and B. Each trace shows electron intensity at a constant energy in terms of variations about a mean intensity at that energy. The encircled crosses indicate zero variation and an average time for the cycle on which they are drawn. The vertical separation of the traces is proportional to reciprocal velocity. The straight lines linking the oscillations provide clear evidence of dispersion, and correspond to a source distance of 42 Mm. [], indicate $\pm 25\%$ variations. Random statistical errors are typically $\pm 4\%$.

auroral oval, Hones *et al.*⁹ have deduced that the degree of stretching is variable, and that on some occasions field lines from approximately the latitude of Kiruna reach out to at least $18 R_E$. Their result is consistent with the degree of stretching deduced from observations of variable dispersion⁸, and therefore our present results indicate that, at the time of the rocket flight, the magnetic field was not severely stretched from its dipole-like configuration.

In view of the location of GEOS 2, close to the equatorial crossing point of magnetic field lines which at times connect with the area of Kiruna, measurements made at the satellite are well suited for comparison with those made at the rocket. An examination of particles of similar energy at GEOS 2, indicates that there are no changes, either inside or outside the loss-cone, corresponding to the longer-period pulsations. It is not yet clear whether 2.2 Hz oscillations in particle flux are detectable by the instruments on GEOS 2. However, VLF emissions measured at GEOS 2 during the rocket flight are noticeably modulated at a frequency close to that of the oscillations in electron intensities at the rocket. These emissions are strongest in a band centred on

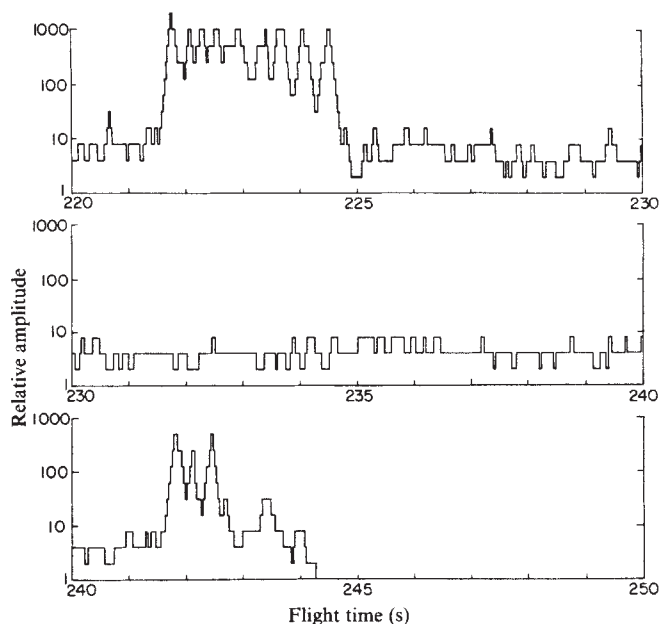


Fig. 3 Intensity of 500 Hz magnetic field fluctuations measured at GEOS 2 by the S300 wave experiment during the rocket flight interval 220–250 s. Oscillations at about 2.8 Hz can be seen between 221 and 225 s, and at about 3.2 Hz between 241 and 243 s.

500 Hz, and are of a type usually referred to as hiss. Figure 3 shows the amplitude of 500 Hz magnetic field fluctuations, measured by experiment S300 on GEOS 2, for the rocket flight interval 220–250 s. Oscillations at 2.8 ± 0.6 Hz are very apparent between 221 and 225 s, and at 3.1 ± 0.6 Hz between 241 and 243 s. Although these do not correspond exactly with events at the rocket they have a similar frequency and occur, like those at the rocket, in irregular bursts. A possible connection between the electron intensity oscillations and VLF activity is not entirely unexpected. Coroniti and Kennel¹⁰ have argued that auroral pulsations are caused by a modulation of the rate of pitch-angle scattering in a diffusion region near the equator. In their model, electrons are scattered by resonance with whistler-mode electromagnetic waves whose growth rates depend on, among other parameters, the amplitude of magnetic micro-pulsations within the diffusion region. Although this is in many ways a promising explanation of auroral pulsations¹¹, there are

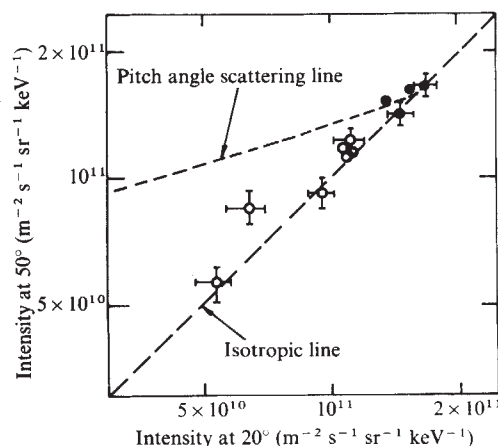


Fig. 4 Relation between intensities at pitch-angles of 50° and 20° for 11.5 keV electrons during interval A: ●, maxima; ○, minima intensities. The pitch-angle scattering line is calculated using a dipole approximation with $L = 5.4$ and a loss-cone of half-angle of 3.44° at the equator.

observations, as on earlier occasions¹², which create difficulties for this theory. Figure 4 shows the relationship between intensities at pitch-angles of 50° and 20° for 11.5 keV electrons during interval A. The broken line, marked pitch-angle scattering, indicates the expected degree of anisotropy for oscillations that are driven by changes in the rate of diffusion of trapped electrons from a reservoir of constant intensity of $1.6 \times 10^{11} \text{ m}^{-2} \text{ s}^{-1} \text{ sr}^{-1} \text{ keV}^{-1}$ into a loss-cone created by atmospheric absorption. Although there is a suspicion that intensities at large pitch-angles exceed those at small pitch-angles, the observations follow more closely the isotropic line than the pitch-angle scattering one, indicating that variations in pitch-angle scattering are not the dominant feature controlling the oscillations. Intensities at other energies during intervals A and B follow closely the behaviour of the 11.5 keV electrons shown in Fig. 4, therefore pitch-angle scattering acting alone cannot explain their modulation. As the nature of the mechanism is unclear at this stage, the extent to which the similarity between the modulation seen at GEOS 2 and the modulation seen at the rocket indicates a real coupling between magnetospheric and ionospheric events remains to be evaluated.

We thank Drs H. Borg, P. Christiansen, P. Gough, A. D. Johnstone, A. Korth and G. Martelli for information and helpful discussions on GEOS 2 particle and wave measurements.

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Observation of flaws in anodic films on aluminium

G. E. Thompson, K. Shimizu & G. C. Wood

Corrosion and Protection Centre, University of Manchester Institute of Science and Technology, PO Box 88, Manchester, M60 1QD UK

A thin compact oxide film forms naturally on aluminium in air and can be thickened by anodizing, to give widely used materials. However, highly localized processes can occur at flaws, which are always present in the films and they can lead to pit development¹ and influence the electrical properties² (dielectric breakdown and rectification). As awareness of the different behaviour at flaws increases, other interesting phenomena, such as electroluminescence³, response to UV radiation⁴ and electrolytic colouring⁵, may be influenced by the activation of pre-existing flaws, or the generation of new flaws. Clearly, further knowledge of this kind is of fundamental and practical importance. Some of the more significant findings relating to the nature, shape and geometry of flaws observed after anodizing in ammonium pentaborate solution are outlined here.

Flaws or lighter regions ~73–200 nm in size, can be observed readily in the barrier films formed to various voltages at constant current density on as-received superpure aluminium (99.99 Al; 0.003 Cu; 0.002 Fe; 0.002 Si wt%) (Fig. 1). Stripped anodic

films show that the pre-existing flaws are now 'decorated' by differently textured film, which spreads into the neighbouring featureless film in an approximately circular front (Fig. 1a, b). This texture changes further with increase in forming voltage and, at 200 V, the local islands have been identified as γ' alumina³ (Fig. 1c). A change in the extent of crystallinity has also been suggested^{6,7}. The corresponding ultramicrotomed sections show that the film formed to 50 V is relatively uniform, although occasional metal ridges protruding into the film from the metal–film interface are evident (Fig. 1d). Further thickening of the film has occurred at 100 V and distinct local regions of differently textured film are evident in approximately the outer half of the film (Fig. 1e). These apparently convex lens-shaped

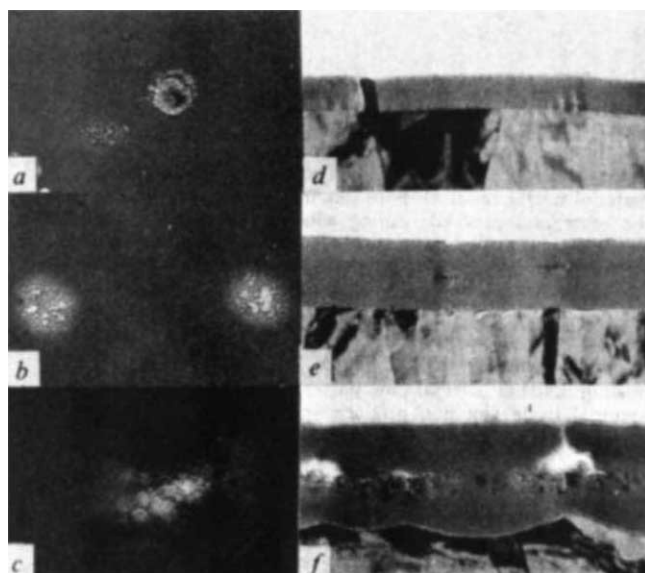


Fig. 1 Transmission electron micrographs of the films formed on as-received aluminium at 50 A m^{-2} in 0.1 M ammonium pentaborate solution at 298 K to various voltages. *a*, Stripped anodic film formed to 50 V, showing the decorated flaws. *b*, Stripped anodic film formed to 100 V showing the different textured appearance of the flaws. *c*, Stripped anodic film formed to 200 V, showing the island of crystalline γ' alumina. *d*, Ultramicrotomed section of the aluminium substrate and the film formed to 50 V. Occasional metal ridges can be seen at the metal–film interface. *e*, Ultramicrotomed section of the aluminium substrate and the film formed to 100 V, showing the sharply defined regions of differently textured film above metal ridges. *f*, Ultramicrotomed section of the aluminium substrate and the film formed to 200 V, showing the marked scalloping at the metal–film interface and the developing regions which have been identified as γ' alumina by electron diffraction³. Magnification $\times 78,000$.

regions are sharply defined and are located above metal ridges at the metal/film interface. A more marked change in appearance is observed at 200 V, where the metal ridges are more clearly defined and the differently textured film is more extensive (Fig. 1f). The locally textured regions of film have spread laterally from their original position centred above the tip of the metal ridge. The islands of crystalline γ' alumina have become damaged during sectioning, but the micrograph suggests that they are thicker at their centres, and the thickness decreases progressively into the neighbouring barrier film. In addition, a narrow channel appears to be present in the barrier film above the crystalline island.

The number of the resultant islands of crystalline film is dependent on substrate preparation and cleaning or electropolishing markedly reduces their population density (from $2 \times 10^{12} \text{ m}^{-2}$ on as received specimens to about 10^9 m^{-2} after

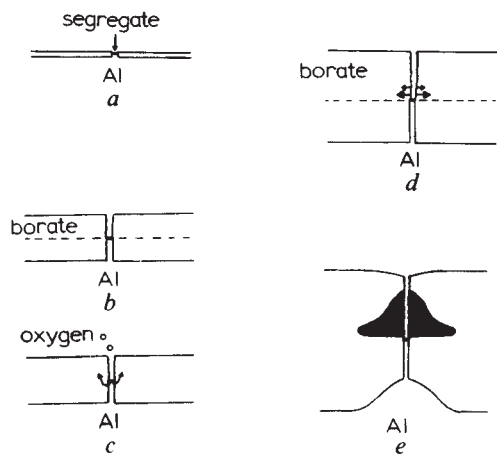


Fig. 2 The development of islands of crystalline film. *a*, An impurity segregate situated at the tip of a metal ridge protruding into the air-formed film. *b*, After anodizing, the segregate is located in the vicinity of the boundary between the borate-containing film material and the relatively pure alumina film. *c*, Gas evolution from the segregates proceeds during anodizing and high local field strengths may cause the aluminium ridge to be removed preferentially, thereby occluding the segregate in the film. *d*, Joule heating allows the development of local temperature rises in the solution-filled channel. The hotter region is situated just above the segregate, and direct heating of the film in this region causes the change in texture. *e*, Crystalline film develops as a result of direct heating and the original flaw passes through the centre of this material. Its development produces shielded regions, which hinder the development of the amorphous barrier film.

electropolishing). This suggests that they develop in the vicinity of surface impurity segregates rather than from effects in the depths of the metal, that is, second phase material, which may be revealed as the metal-film interface recedes during anodizing. Favoured sites for segregation of impurities, such as iron and copper, exist on rolled surfaces, that is, in the vicinity of metal ridges and troughs associated with rolling lines. Growth of the air-formed film, which is more voluminous than the aluminium consumed, then creates a distribution of flaws, comprised of metal segregates situated at the bottoms of channels in the film, which are located at the tips of metal ridges protruding into the film from the metal-film interface (Fig. 2*a*). A characteristic air-formed film is also likely to form over the segregates.

During anodizing, thickening of the air-formed film occurs by the usual growth processes involving ionic migration of Al^{3+} outwards and OH^- or O^{2-} ions inwards⁸. SIMS data (G.E.T. and G.C.W., unpublished work) suggest similar anion and cation transport numbers, leading to an inner relatively pure alumina film and deposition of an outer borate-containing film. However, film formation does not proceed directly above the segregates where an alternative anodic process, oxygen evolution occurs or in the shielded regions directly below the segregates. This manner of film growth results in the segregates being located at the tips of metal ridges protruding into about one-half of the total film thickness (Fig. 2*b*). Gas evolution from the segregates tends to maintain the channels above them open by mechanically disrupting the outer film, which attempts to bridge over them. Unless the shielding of the aluminium ridges is effective, high local field strengths may result in the consumption of this metal in lateral film growth, and the possible inclusion of some of the segregate into the film (Fig. 2*c*).

The areas of the bases of the segregates are extremely small compared with the macroscopic aluminium substrate and large effective current densities can be directed to these relatively low resistance pathways, with resultant large temperature rises due to local Joule heating effects. This is unlike the situation suggested to occur during pitting, at localized sites, where salt film formation proceeds rapidly and the temperature at the pit

surface remains essentially unchanged⁹. As the film thickens further, more current is focused into these preferred regions, which is confirmed by the more ready observation of gas evolution at high cell voltages, with greater local heating effects. Heat is dissipated into the aluminium substrate and bulk solution, but because the segregates are located above the tips of relatively narrow metal ridges, it is likely that the rate of heat flow into the substrate is limited and the hottest zone in the solution-filled channel is situated just above the segregate (Fig. 2*d*).

Direct heating¹⁰, or hydrothermal treatment¹¹, increases the extent of crystallinity of anodic films, at a rate dependent on their composition. Similar texture changes to those evident here initially have been observed during electron beam heating of anodic films¹², suggesting that they may be promoted by direct heating. However, there is presently little direct information about the magnitude of the local temperature rises. Figure 2*d* shows that the hot zone is adjacent to the borate-containing film material, which implies that this film material undergoes the more rapid change in texture and ultimate increase in crystallinity.

In the present situation the local heating is thought to develop continuously rather than in discrete bursts, which are more commonly associated with electron avalanching and dielectric breakdown. Direct heating promotes the increase in the extent of crystallinity of the material adjacent to the hotter regions of the channel and, as the barrier film thickens, the hot regions spreads up the channel resulting in an increase in the volume of the resultant crystalline island. The shape of the crystalline region is explained by the hotter region remaining just above the segregate, but with further barrier film thickening temperature rises adjacent to regions further up the channel promote local crystallization. The actual stage at which crystallinity appears is a compromise between the size and population density of the flaws and the film thickness, which govern the effective current density and local temperature rises generated. Anodizing in borate solution favours development of the islands³, which suggests that the eventual crystallization process may be dependent on electrolyte conductivity and buffering capacity and/or film composition. Furthermore, it is not clear whether borate species incorporated in the film aid this process directly by reducing the crystallization temperature or migrate from the hotter regions during crystallization and whether borate species act as glass network formers or modifiers. However, the eventual development of these islands, which hinders the growth of the normal amorphous barrier film results in the development of scalloped metal regions at the metal-film interface (Fig. 2*e*).

Note also that electroluminescence is observed during the growth of these films in borate solutions. The significance of flaws, film composition and the development of crystalline film is in the stages of elucidation and will be reported elsewhere.

In conclusion, it is suggested that iron and/or copper impurities are responsible for the behaviour observed during anodizing in borate solution. They remain at the tips of metal ridges located at the bases of channels within the films and local temperature rises associated with Joule heating effects give rise to the development of crystalline film.

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The structure and energetics of the conduction plane in $\text{Na}\beta\text{Al}_2\text{O}_3$

J. R. Walker & C. R. A. Catlow

Department of Chemistry, University College London,
20 Gordon Street, London WC1H 0AJ, UK

An atomistic computer simulation study of the superionic conductor $\text{Na}\beta\text{Al}_2\text{O}_3$ is described here. This material is a layer structured compound in which highly mobile sodium ions are located in 'conduction planes' between spinel structured Al_2O_3 blocks¹. Although we have investigated structural properties of the conduction plane, which have been widely studied by diffraction techniques, our results are also important to transport properties of the compound. We demonstrate that a very flat potential surface governs the distribution of Na^+ ions in the conduction plane. This finding is clearly compatible with superionic properties of the compound but also has consequences for analysis of structural data. Our calculations confirm that O^{2-} interstitials are stabilized by large relaxations of neighbouring lattice ions to give the cluster proposed earlier². Defects in the conduction plane of $\beta\text{-Al}_2\text{O}_3$ are shown to be generally stabilized by large relaxations of surrounding ions, which favour defect formation in the compound, and which, therefore, cause the gross deviations from stoichiometry observed in $\beta\text{-Al}_2\text{O}_3$. Our results suggest a 'fluid' conduction plane structure (a fluidity which extends to the neighbouring spinel block ions) with only weak localization of the conduction ions into specific sites and strong stabilizing relaxations of conduction and spinel block ions around any defect species.

Two types of calculation are discussed. In the first, lattice energy calculations on stoichiometric $\text{Na}\beta\text{Al}_2\text{O}_3$ for a variety of sodium ion distributions, use the general lattice energy code, PLUTO³, previously applied successfully to complex problems in the field of non-stoichiometry^{4,5}. In the second, calculations of the energies of formation of defects within the conduction plane of stoichiometric $\text{Na}\beta\text{Al}_2\text{O}_3$ use another general computer code, the HADES III⁶, which can be applied to all crystal symmetries. The HADES code is an improvement on earlier theoretical studies of Wong *et al.*, which omitted relaxation effects.

Both types of calculation require specification of the relevant interionic potentials. Shell model potentials (see refs 3, 6) are

used. Thus to model $\text{Na}\beta\text{Al}_2\text{O}_3$ we take parameters from the lattice potential of Al_2O_3 (refs 4, 6) which are supplemented by a $\text{Na}^+\text{-O}^{2-}$ potential obtained from a study of the crystal properties of Na_2O ; for the shell parameters of the Na^+ ion we use the values reported in ref. 7. The present calculations were prompted by their successful application in other systems^{5,6,8}.

Three important cation sites in the conduction plane of $\text{Na}\beta\text{Al}_2\text{O}_3$ have been identified. Figure 1 shows the positions known as the Beevers-Ross, anti-Beevers-Ross and mid-oxygen sites. We have, therefore, performed three lattice energy calculations; in each case all the Na^+ ions were situated at one of the three sites. The Beevers-Ross configuration is found to have the lowest lattice energy. The other results are summarized in Table 1.

Table 1 Energies of Na^+ ion configurations

Configuration	Energy* (eV)
Mid oxygen	0.39
Anti-Beevers-Ross	0.15

* Energies are given per Na^+ ion and with respect to the Beevers-Ross configuration.

Several important consequences follow from these calculations. First, the Beevers-Ross sites are the most energetically favourable for the incorporation of Na^+ ions—a result in line with experimental observation⁹. But the differences between the energies of the Na^+ at this site and other configurations are very small—of the same order of magnitude as kT for elevated temperatures (500–1,000 K). The existence of such an exceptionally flat potential surface provides a qualitative explanation of the superionic nature of $\text{Na}\beta\text{Al}_2\text{O}_3$. Little resistance to Na^+ motion can be provided by the alumina framework, and the conduction mechanism and activation energy will be largely controlled by the interactions between the Na^+ ions. Second, the flatness of the potential surface will result in very large and anharmonic thermal parameters in any refinement of diffraction data on the material—a conclusion of obvious importance in structural studies of $\text{Na}\beta\text{Al}_2\text{O}_3$. Third, our present theoretical methods contain limitations. If the potential energy of the Na^+ ion between different sites in the conduction plane varies only by $\sim kT$, then transport properties of the solid cannot be adequately modelled by 'static' calculations of the type discussed here because the correlation between the motions of several species cannot be modelled by our technique which concentrates on isolated defect species. Dynamical simulations as reported by

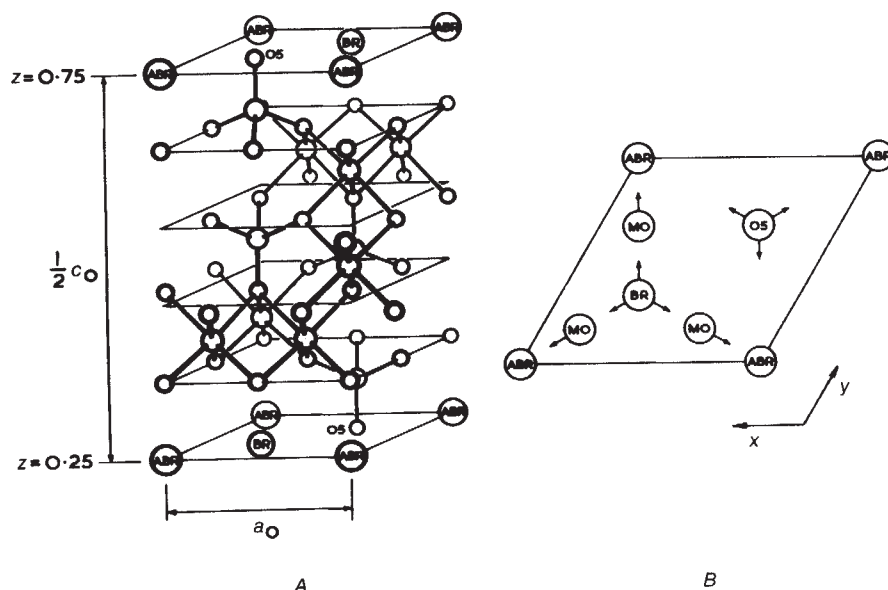


Fig. 1 The structure of $\beta\text{-Al}_2\text{O}_3$ with Beevers-Ross (BR), anti-Beevers-Ross (ABR) and mid-oxygen (MO) sites marked. A, 3D Structure. $\circ$, Al^{3+} ; $\bullet$, O^{2-} . B, Section of structure through conduction-plane.

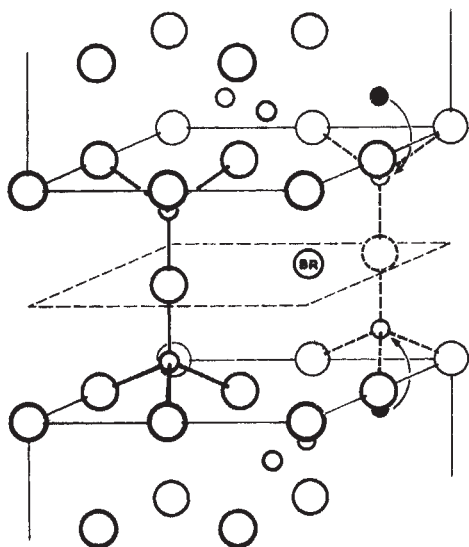


Fig. 2 Oxygen interstitial in conduction plane. The interstitial (○) is stabilized by the movement of the Al^{3+} ions (●) as shown. A bridging oxygen ion is also shown.

Dixon and Gillan¹⁰ for the superionic fluorite materials will be required.

Excess oxide ions in non-stoichiometric $\beta\text{-Al}_2\text{O}_3$ are incorporated in the conduction plane. Recent diffraction studies have suggested that these O^{2-} ions are stabilized by the displacement of two neighbouring aluminium ions from their lattice positions into 'interstitial' sites above and below the conduction plane; Fig. 2 illustrates the resulting complex. To test the feasibility of these proposals, we performed calculations using the HADES III program first on the conduction-plane O^{2-} interstitial, and second on the interstitial with both neighbouring displaced Al^{3+} ions. We found that displacement of both ions resulted in a stabilization energy of 1.0 eV. Our results confirm the Roth model. They also indicate a tendency for exceptionally large displacement of lattice ions around 'defects' in $\beta\text{-Al}_2\text{O}_3$.

We have performed calculations using the HADES III program of vacancy and interstitial energetics in $\beta\text{-Al}_2\text{O}_3$, and found, remarkably, that the formation of all defect species is energetically favourable. The result is not attributable to inadequacies in our lattice model, as the calculations converged successfully, without excessive ion dipoles developing. Rather, the favourable defect energies are due to the ability of the conduction plane and neighbouring spinel block ions to relax extensively around charged defects—a feature of the defect chemistry of $\beta\text{-Al}_2\text{O}_3$ already shown by the structure of the oxide interstitial, but which our work suggests is general to this material.

It is also clear that if defect formation is a favourable process in $\text{Na}\beta\text{Al}_2\text{O}_3$, then the stoichiometric compound would not be expected to be stable; the compound will disproportionate into a defective non-stoichiometric compound and Al_2O_3 . Although our discussion cannot yet be more quantitative, we believe that we have identified the essential feature of $\text{Na}\beta\text{Al}_2\text{O}_3$ responsible for its unusual stoichiometry, namely the exceptional stability of defects which in turn arises from the very large ion relaxations possible around conduction plane species.

Our results suggest that the unique features of $\beta\text{-Al}_2\text{O}_3$ can be attributed to two related factors: the flatness of the sodium ion potential surface in the conduction plane; and the tendency for large ion relaxations around defect species in the plane. This 'fluidity' of the conduction plane is obviously the basic cause of the superionic properties of $\beta\text{-Al}_2\text{O}_3$. However, it raises serious problems for the detailed modelling of the material. Static simulations cannot yield information beyond the general guidance which the present results have provided. Molecular

dynamic simulations are necessary. Preliminary results have been reported by de Leeuw¹¹, but detailed simulations would be of great interest.

We are grateful for financial support from AERE Harwell, and the Commission of the European Communities. We thank the SRC for permission to use the CRAY-1 computer at the Daresbury Laboratory.

Received 21 April; accepted 6 June 1980.

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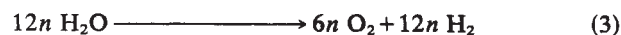
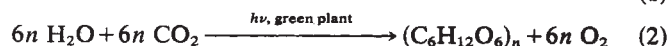
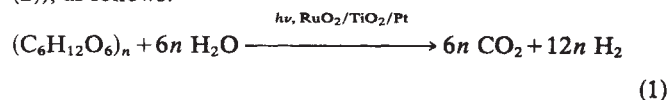
Conversion of carbohydrate into hydrogen fuel by a photocatalytic process

Tomoji Kawai & Tadayoshi Sakata

Institute for Molecular Science, Myodaiji, Okazaki 444, Japan

Attempts have been made to find green plants which produce low-molecular weight hydrocarbons^{1,2} and to find seaweeds which produce hydrogen from water utilizing solar energy^{3,4}. These attempts are aimed at finding methods of making use of the photosynthetic process in plants for direct production. Most green plants, however, synthesize carbohydrates, such as sugar, starch and/or cellulose, from water and carbon dioxide. The C_4 plants⁵, such as corns and sugar cane, grow rapidly, utilizing solar energy with ~1% efficiency for the fixation of CO_2 . This value is 10 times larger than that of the average efficiency of photosynthesis of plants, 0.1%. However, the carbohydrates produced by these plants cannot be used directly as fuel. Here we show a new route for the conversion of carbohydrates into hydrogen (a clean fuel in the hydrogen energy system), taking advantage of the photocatalytic process. We found that the irradiation of the carbohydrates, not only sugar or starch but also cellulose, in the presence of water and a $\text{RuO}_2/\text{TiO}_2/\text{Pt}$ photocatalyst powder leads to the efficient production of hydrogen gas.

This new process is expressed as equation (1) together with the photosynthesis of carbohydrates by green plants (equation (2)), as follows:



Here, $(\text{C}_6\text{H}_{12}\text{O}_6)_n$ represents sugar (saccharose) ($n = 2$), starch ($n \approx 100$) or cellulose ($n \approx 1,000\text{--}5,000$) after hydrolysis.

Accordingly, the overall reaction results in the decomposition of water to produce hydrogen (equation (3)), the CO_2 being recycled. The reaction (1) proceeds almost without loss of energy (heat of combustion: $688 \text{ kcal mol}^{-1}$ for $(\text{C}_6\text{H}_{12}\text{O}_6)_{n=1}$ and $680 \text{ kcal mol}^{-1}$ for 12H_2).

The typical experimental conditions are as follows: the powders of RuO_2 (Rare Metallic), TiO_2 (Katayama Kagaku) and Pt (Engelhardt) (weight ratio = 10:100:5) were mixed and ground in an agate mortar, and this powder was suspended in

neutral water (40 ml) in a Pyrex glass bulb (280 ml). Then a sugar (600 mg, Nissin Seito pure white sugar) or a soluble starch (120 mg, Katayama Kagaku) was dissolved in the solution and the bulb was deaerated and illuminated by a 500 W Xe lamp. For the reaction of cellulose, a filter paper (Toyo Filter) was cut into pieces and put into neutral water or a 6 M NaOH water solution with the photocatalyst forming alkali-cellulose. The gases evolved were collected and analysed by a mass spectrometer, and the amounts of the gases were determined with a manometer with traps at -197 , -78 and -10 °C, as described previously⁶.

When the photocatalyst suspension in water with the substrate was illuminated, gas bubbles were vigorously generated out of the depth of the solution near the illuminated area. The bubbling stopped when the light was turned off. The evolved gases were mainly H_2 and CO_2 , with very small amounts of CH_3OH and C_2H_5OH (less than 0.2% of the total amount). Table 1 summarizes the amounts of hydrogen evolved from sugar, starch or cellulose with water, and Fig. 1a shows the evolution of H_2 and CO_2 from sugar plotted against irradiation time. The ratio of the formation rate of H_2 to CO_2 was 3:1 to 4:1 at the initial stage, becoming about 2:1 in the stationary state. No CO , CH_4 and O_2 were evolved. When NaOH was put into the solution to form 6M NaOH solution during the reaction, only H_2 was evolved (Fig. 1a), and the CO_2 produced was absorbed in the solution presumably as Na_2CO_3 or $NaHCO_3$. Also with the cellulose in 6M NaOH aqueous solution, pure hydrogen was evolved (Table 1). The quantum yields of the H_2 production determined by monochromatic irradiation at $\lambda = 380$ nm for sugar, starch and cellulose were 1.2, 0.8 and 0.3% respectively in neutral water and 1.5, 1.3 and 1.0% respectively in 6M NaOH. These quantum yields, using the $RuO_2/TiO_2/Pt$ photocatalyst, were 100 times larger than those obtained with TiO_2 alone (Table 1). To confirm that no detectable amounts of other products were formed in the solution at the end of the reaction, an experiment was carried out with a small amount of sugar (13 mg, corresponding to 1 mmol of H_2 gas), using a Hg-ultra-high pressure lamp (500 W, Philips) which emits intense UV light ($\lambda < 320$ nm was cut by a Pyrex glass filter), instead of the Xe lamp. The sugar was then completely decomposed within 18 h producing only the corresponding H_2 and CO_2 gases (Fig. 1b). These results show that the reaction (1) occurs successfully in this experimental condition at room temperature, simply producing gaseous H_2 and CO_2 . Surprisingly, the hydrogen was evolved not only from water and sugar, a relatively large molecule, but also from water and starch or

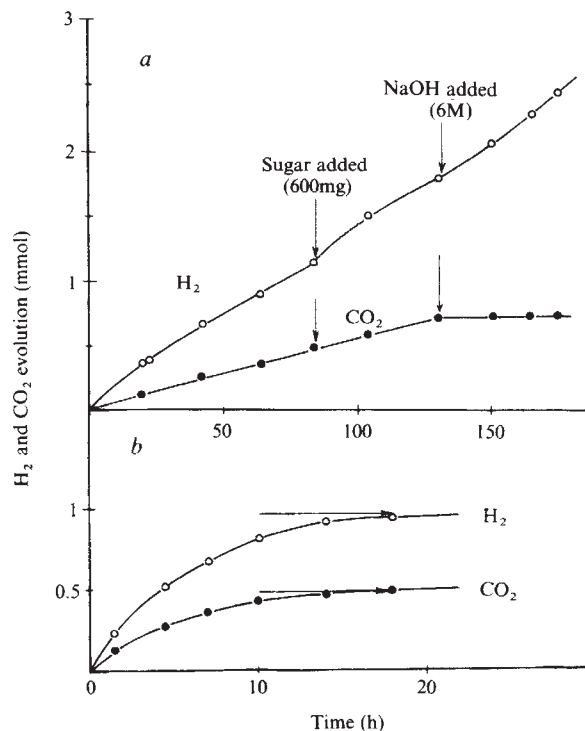


Fig. 1 a, H_2 and CO_2 evolution from sugar in water versus the irradiation time. Experimental conditions: sugar (600 mg), H_2O (40 ml), $RuO_2/TiO_2/Pt$ (300 mg), 500 W Xe irradiation. Additional sugar (600 mg) and NaOH (9.6 g) were put into the solution at 85 and 130 h after the start of the reaction, respectively. b, H_2 and CO_2 production with the irradiation time from a small amount of sugar. Experimental conditions: 500 W Hg-ultrahigh-pressure lamp, sugar (13 mg), H_2O (40 ml), $RuO_2/TiO_2/Pt$ (150 mg). Arrows ($\rightarrow$) indicate the estimated amounts of H_2 and CO_2 which are produced from 13 mg of sugar according to equation (1).

cellulose, the stable macromolecule which consists of about a hundred to thousands of molecules of glucose.

The mechanism of the reaction could be explained by regarding the $RuO_2/TiO_2/Pt$ powder as an electrochemical microcell, as follows: the light of energy larger than the band gap of TiO_2 (3 eV) produces electron-hole pairs; then the separated electron and holes can make redox reactions at the surface of the particle with the molecules in the solution (Fig. 2). This particular $RuO_2/TiO_2/Pt$ photocatalyst is very active in reaction (1) (100 times more active than TiO_2 alone), presumably because RuO_2 is one of the best electrode materials for the oxidation of water⁷ and Pt for hydrogen evolution⁸. As carbohydrate is expressed as

$(CH_2O)_n$ and is an assembly of $(H-C-OH)_n$ which has alco-

holic $-OH$ groups, we chose CH_3OH and $HCHO$ as the model C_1 compounds, and examined their photocatalytic reactions with water on the $RuO_2/TiO_2/Pt$ photocatalyst. It is already known that CH_3OH is photooxidized to form $HCHO$ on a single crystal TiO_2 photoanode⁹ ($CH_3OH + 2p \rightarrow HCHO + 2H^+$). In our experiment using $RuO_2/TiO_2/Pt$ powder, the CH_3OH was oxidized into $HCHO$, and simultaneously, H^+ was reduced to form H_2 (Quantum yield $\approx 44\%$). It was also observed that $HCHO$ with water was finally converted into H_2 and CO_2 ($H_2:CO_2 = 2:1$) on this photocatalyst ($HCHO + H_2O \rightarrow 2H_2 + CO_2$). Consequently, the carbon chains of carbohydrates in water would be successively oxidized by the hole at the oxidizing site of the catalyst to form H^+ and CO_2 , while the electrons would reduce H^+ at the reducing site to form H_2 , similar to the case of CH_3OH and $HCHO$. The larger amount of H_2 production at the beginning of the reaction would be due to the dehydrogenation of the carbohydrate to form $>C=O$, $-CH=O$ or $-COOH$ functional groups at the initial stage.

Table 1 H_2 and CO_2 evolution from water and sugar, starch or cellulose on the $RuO_2/TiO_2/Pt$ photocatalyst suspended in neutral water and 6M NaOH solution, irradiated by 500 W Xe lamp

Reactant	Quantum yield at $\lambda = 380$ nm	
	H_2 ($\mu\text{mol}/20$ h)	CO_2 ($\mu\text{mol}/20$ h)
Sugar + H_2O	280	133
Starch + H_2O	204	96
Cellulose + H_2O	70	42
H_2O	4	*
Sugar + 6M NaOH	341	—
Starch + 6M NaOH	320	—
Cellulose + 6M NaOH	244	—
Sugar + H_2O with TiO_2 catalyst	2	1.2

Experimental conditions: sugar (600 mg), starch (120 mg), cellulose (120 mg), $RuO_2/TiO_2/Pt$ photocatalyst (300 mg), H_2O (40 ml), 6M NaOH aqueous solution (40 ml). All the values fluctuated within at most $\pm 10\%$. The rates of gas evolution represent the values at 50 h after the beginning of the irradiation. The quantum yield means the number of electrons used for the H_2 evolution divided by the number of incident photons, taking into consideration that two electrons produce one H_2 molecule.

* Oxygen ($1.6 \mu\text{mol}$ per 20 h) was simultaneously evolved.

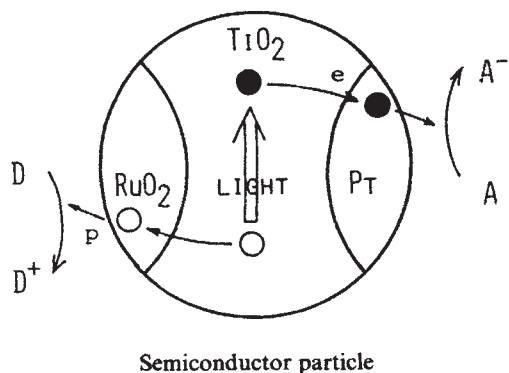


Fig. 2 A schematic figure of the $\text{RuO}_2/\text{TiO}_2/\text{Pt}$ particle. $\circ$, Hole (p) in the valence band of TiO_2 ; $\bullet$, electron (e) in the conduction band. A and D represent acceptor and donor, respectively, of electrons in the solution. In reaction (1), carbohydrate and proton in the solution play the roles of D and A, respectively.

Note also that this $\text{RuO}_2/\text{TiO}_2/\text{Pt}$ photocatalyst can decompose water directly into H_2 and O_2 with a quantum yield of 0.02% at $\lambda = 380 \text{ nm}$ (Table 1).

As solar energy conversion into chemical energy by a man-made device with efficiency of $>1\%$ is not cheap or easy at this stage, the use of the photosynthetic power of green plants for making fuels is still effective¹. In Brazil, a project is under way in which sugar taken from rapidly growing sugar cane is fermented into ethanol for use as a fuel, using bacteria^{1,10}. The fermentation, however, is a delicate procedure, and occasionally an acid is formed instead of alcohol. In our method, the capture and the fixation of dilute CO_2 is left to the plants, and the produced carbohydrates are artificially converted into H_2 and CO_2 using the photocatalytic reactions on the stable inorganic semiconductor powder. This process may be called 'photoinduced fermentation'. The CO_2 evolved can be collected and may be fed back to the plants as concentrated CO_2 gas. In the concentrated NaOH solution, we can get pure hydrogen gas (Fig. 1a) and simultaneously Na_2CO_3 or NaHCO_3 , important substances for the chemical industry. Furthermore, the rates of H_2 production from carbohydrates were greatly enhanced in 6M NaOH solution compared with that in neutral aqueous solution (Table 1). Another method for the production of pure hydrogen, utilizing reaction (1) in a photoelectrochemical cell is now under investigation: the carbohydrate with water is oxidized at a semiconductor photoanode to form CO_2 in one cell, with H_2 production from water at the cathode in the other cell.

Using the strong oxidizing power of the $\text{RuO}_2/\text{TiO}_2$ photocatalyst powder, we have demonstrated the photoinduced water-gas reaction with formation of H_2 from water and solid carbon⁶, the direct decomposition of water into H_2 and O_2 (refs 11, 12) and, in this study, the decomposition of carbohydrate by $\text{RuO}_2/\text{TiO}_2/\text{Pt}$ to produce H_2 . This method may also be applied to the decomposition of excrements containing cellulose, protein and fat, accompanied by the production of H_2 as a fuel. We are also interested in the possibility of the decomposition and scavenging of abandoned carbon compounds in nature (such as corpses of animals) using a semiconductor photocatalyst (Fe_2O_3 , WO_3 and so on) instead of bacteria.

Received 4 March; accepted 15 May 1980.

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Trace element abundances in basalts of Nauru Basin

R. Batiza*, R. L. Larson†, S. O. Schlanger‡, S. A. Shcheka§ & H. Tokuyama||

*Department of Earth and Planetary Sciences, Washington University, St Louis, Missouri 63130

†Lamont-Doherty Geological Observatory of Columbia University, Palisades, New York 10964

‡Hawaii Institute of Geophysics, University of Hawaii at Manoa, Honolulu, Hawaii 96822

§Far East Institute of Geology, USSR Academy of Sciences, Vladivostok USSR

||Ocean Research Institute, University of Tokyo, Nakano, Tokyo 164, Japan

Leg 61 of the Deep Sea Drilling Project (DSDP) was concerned with drilling a single continuously cored multiple re-entry hole at site 462 in the Central Nauru Basin (Fig. 1). Preliminary results of this drilling, which penetrated more than 1 km beneath the sea floor, were presented earlier^{1,2}. One major result was the discovery of a late Cretaceous off-ridge volcanic/intrusive complex of basaltic composition and great thickness ($>500 \text{ m}$). We now present trace element abundance data for these basalts. Results of the drilling provide further support for a relatively long-lived thermal and magmatic event in the late Cretaceous resulting in voluminous and widespread magmatism in the central and western Pacific consistent with earlier suggestions³⁻⁵. The trace element data show that most of the rocks produced during this event have trace element characteristics intermediate between those of normal and transitional mid-ocean ridge basalts (N- and T-type MORB)⁶ and different from Hawaiian basalts⁷. These results indicate that basalts which are depleted in light rare earth elements (LREE) relative to the heavy REE may, in certain conditions, be erupted as voluminous intra-plate eruptions far from active ridge crests.

DSDP site 462 is located in the Central Nauru Basin in a region of low topographic relief and bottom depths of $\sim 5,100 \text{ m}$ (ref. 1) and lies on magnetic anomaly M-26 ($\sim 155 \text{ Myr BP}$)⁸⁻¹⁰. The Nauru Basin is bounded on the west and south by the Ontong-Java plateau¹¹⁻¹³, on the east and north by the Gilbert and Marshall ridges^{3,5,10,14,15} and on the north-west by the Caroline Ridge¹⁵⁻¹⁸ (Fig. 1). It is a region of well developed Mesozoic magnetic anomalies and since the mid-Cretaceous has been the site of turbidite deposition from the surrounding elevated regions¹. Palaeomagnetic data show that the Nauru Basin basalts are all normally magnetized, indicating that they were largely emplaced during the Cretaceous long normal event which lasted from 109 to 80 Myr BP. As the age of the Pacific crust at this site is about 155 Myr BP, this magmatic event occurred off-ridge on oceanic crust at least 46 Myr old^{1,8,19}.

Based on on-board petrographic and X-ray fluorescence analyses for several major elements¹ and later shore-based chemical analyses of several hundred samples, it is clear that the basalts produced during the off-ridge magmatic episode in the Nauru Basin are mineralogically and chemically similar to MORB^{1,6}. They are quartz and olivine-hypersthene normative and have mineralogy and major trace element characteristics which are very similar to many types of oceanic ridge basalt (see Table 1). However, they differ significantly and systematically in the following respects: (1) Nauru Basin basalts have higher FeO^*/MgO ratios for a given TiO_2 content than N-type MORB. For example, at TiO_2 contents of 1.0 wt %, FeO^*/MgO is 1.3–1.5 compared with 0.8–1.0 for N-type MORB^{6,20}. Trends of FeO^*/MgO and TiO_2 wt % for Nauru Basin basalts are parallel to trends for N-type MORB but displaced to higher FeO^*/MgO ratios. (2) Spinels in Nauru Basin basalts have lower $\text{Mg}/\text{Mg} +$

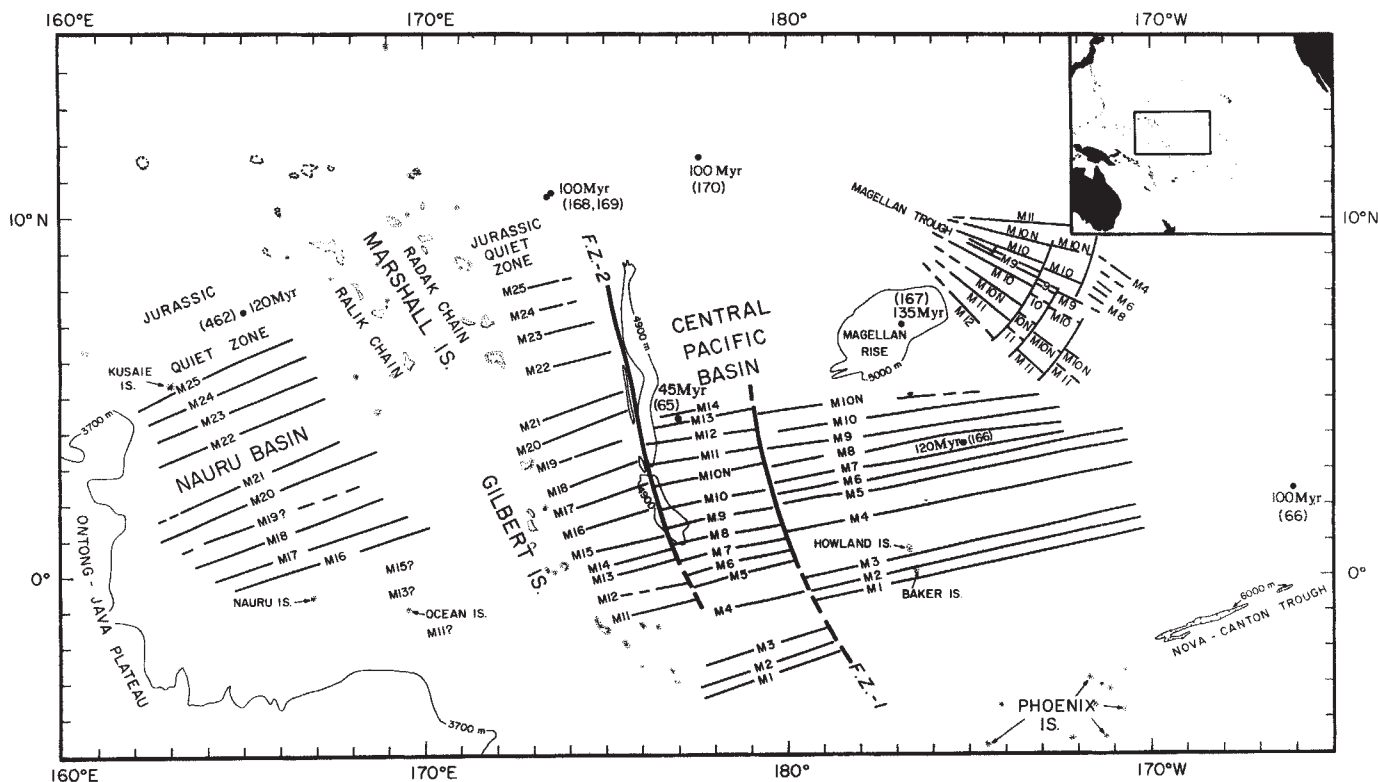


Fig. 1 Bathymetry of the western and central Pacific²³ showing the location of DSDP sites 462 and 169.

Fe^{2+} ratios (0.49–0.54) and higher Cr/Cr + Al ratios (0.54–0.73) than N-type MORB (0.6–0.8 and 0.4–0.6, respectively)²¹ and these co-exist with olivine of generally lower Mg number (100 Mg/(Mg + Fe^{2+}) of olivines is 0.83–0.84) than found in N-type MORB. (3) Nauru Basin basalts have higher La/Sm ratios at given Sm contents than N-type MORB, but lower La/Sm ratios than transitional MORB⁶ and Hawaiian tholeiitic basalts⁷ (see Figs 2, 3). Abundances of Sc, Cr, Co, Ni, Sr, Hf and Zr fall within the range of abundances for MORB (Table 1). Thus, Nauru Basin basalts are depleted in LREE as are N-type MORB, but the level of depletion of LREE is significantly and systematically less than that of N-type MORB.

The geological evolution of the central and western Pacific during the Mesozoic is apparently quite complicated and may involve very rapid spreading rates from several relatively short-lived ridge crest segments, numerous ridge jumps and creation of thick volcanic piles^{3,5,22,23}. The formation of abundant seamount and island chains^{15,24}, guyots²⁵ and high standing plateaus^{5,13} is thought to have occurred in late Mesozoic time but the relationship between this widespread volcanism, vertical movements and plate geometrics and evolution remains problematic^{3-5,10,26}.

A possible clue to the nature of volcanic/tectonic evolution of the western and central Pacific is provided by the widespread occurrence of off-ridge basalts with trace-element abundances like those of the Nauru Basin elsewhere in the region. Basalts which are depleted in LREE but which have unusually high La/Sm ratios (relative to the N-type MORB) have been encountered in DSDP holes 169 (ref. 27), 289 (ref. 28) and 317 (ref. 29) drilled during Legs 17, 30 and 33. These basalts occur either as sills or as lava flows on plateau regions and contrast chemically with volcanic rocks of broadly similar age which form the seamounts and seamount chains of the western and central Pacific^{24,29}, all of which are alkalic and more enriched in LREE. It is possible that much of the crust in the area of the Darwin Rise³, within which the Nauru Basin and other DSDP sites that encountered off-ridge basalt with MORB trace element abundances all lie, is pervasively intruded.

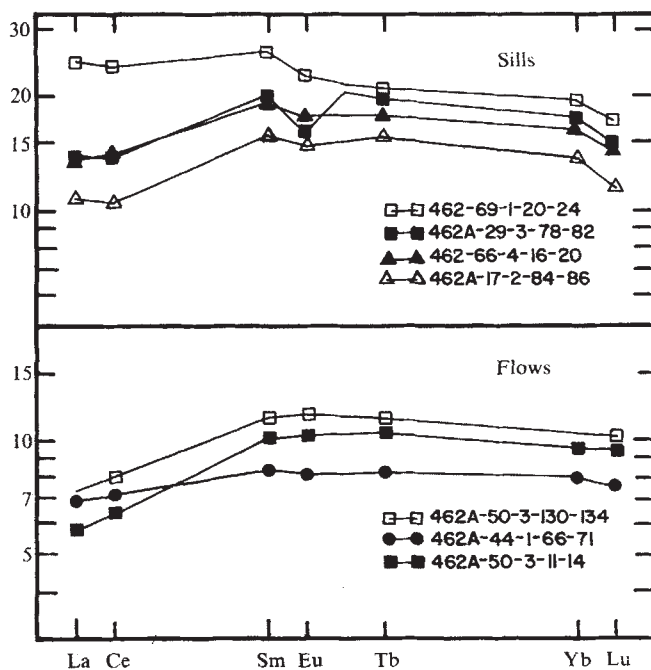


Fig. 2 Representative chondrite-normalized REE abundance patterns for several samples drilled during DSDP Leg 61. The sills (with interlayered sediment) comprise the upper portions of the Nauru Basin igneous complex and the flows occupy the lower portions of the section. Analyses were carried out by instrumental neutron activation analyses³². Rare earth and other trace element data will be reported elsewhere³³ but are available before then from R.B.

Table 1 Representative chemical analyses

Oxide (wt %) or element (p.p.m.)	Nauru Basin			Ontong-Java Plateau
	462-64- 1-8-11	462A-20- 1-115-121	462A-72- 3-21-23	289-132- 4-6-10
SiO ₂	49.78	47.50	49.24	49.4
TiO ₂	1.52	1.62	1.02	1.6
Al ₂ O ₃	14.76	14.69	15.33	14.9
Fe ₂ O ₃	3.62	2.88	3.52	4.1
FeO	9.07	10.15	7.30	7.5
MnO	0.21	0.26	0.19	0.14
MgO	7.01	7.09	7.70	6.6
CaO	9.44	11.14	12.29	10.6
Na ₂ O	2.19	2.26	1.95	2.3
K ₂ O	0.12	0.05	0.05	0.34
P ₂ O ₅	0.17	0.18	0.13	0.14
H ₂ O ⁺	1.08	0.82	0.99	2.2
H ₂ O ⁻	0.67	1.08	0.81	
Total	99.64	99.72	100.52	99.82
La	4.29	4.13	2.59	4.45
Ce	13.1	11.53	7.41	11.77
Sm	3.33	3.18	2.19	3.59
Eu	1.29	1.11	0.81	1.12
Tb	0.95	0.82	0.61	0.85
Yb	3.78	2.98	2.28	3.09
Lu	0.55	0.45	0.35	0.48
Sc	51	41	45	46
Cr	228	179	313	298
Co	51	44	46	38
Hf	2.96	2.51	1.54	2.40

Analyses of two sills from hole 462, core 64 and hole 462A, core 20 and 1 flow from hole 462A, core 72. Analysis of a basalt from DSDP Leg 30 is given for comparison with the DSDS Leg 61 sills. Major element chemistry of the Leg 30 sample is from Stoesser²⁶ (sample 289-132-4-27). Major element analyses of Leg 61 samples were done by wet chemical techniques and trace elements by instrumental neutron activation analysis³².

If this speculation is correct, very large portions of the Pacific may have been constructed by a combination of normal oceanic crustal creation processes (at ridges) together with voluminous off-ridge additions of basaltic material of broadly similar chemical composition. Perhaps coincidentally, the Caribbean flood basalts encountered during Leg 15 of DSDP have nearly identical trace element chemistry to the Nauru Basin and other basalts described previously³⁰ and the crustal ages and structures inferred from geophysical studies are also similar^{9,31}. This may indicate that conditions leading to the generation of large volumes of intra-plate basalt chemically similar to MORB were not unique to the Pacific.

The major conclusions from the trace element data of Nauru Basin basalts and interpretation of these data in the light of volcanic and tectonic evolution of the central and western Pacific

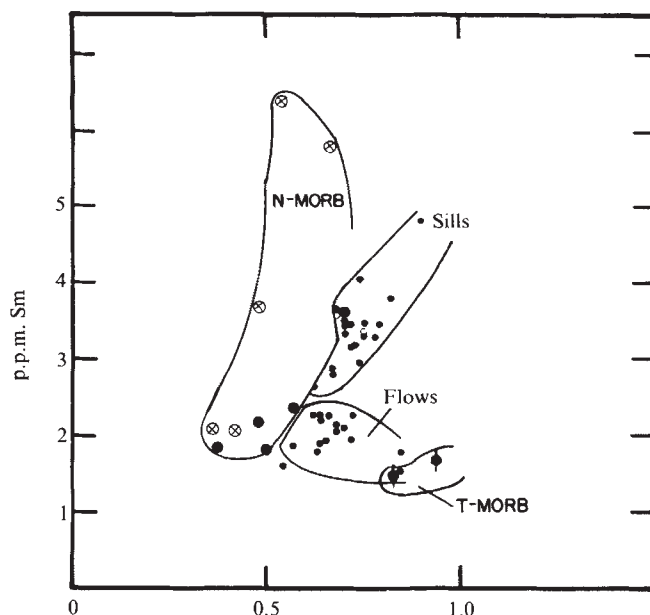


Fig. 3 (La/Sm) chondrite normalized enrichment factor as a function of Sm abundance for Leg 61 lavas (small dots). Also shown, for comparison, are normal mid-ocean ridge basalts (MORB) from the Siqueiros Fracture Zone³⁴ (circled crosses), analyses from Sun *et al.*⁶ (large dots) and analyses from the Ontong-Java plateau (small circles). Note the clear separation of Leg 61 sills from flows and the intermediate La/Sm ratios of Leg 61 basalts. They occupy a field between normal and transitional types of MORB.

are as follows: (1) The Nauru Basin volcanic complex is composed of basalt which has trace element characteristics that are intermediate between N- and T-types of MORB. (2) Basalt of this composition is widely distributed throughout the western and central Pacific in sills and aseismic plateaus, but it is not known if this distribution is continuous. Basalt of similar chemistry is also present in the Caribbean crust. (3) More tentatively, it may be concluded that the wide distribution of off-ridge basalt chemically similar to MORB in the crust of the western and central Pacific is an indication of unusual processes of crustal origin and evolution in this region. Clearly, though, much more information is required before the nature of such processes is determined.

We thank Larry Haskin for allowing use of facilities for trace element determinations. This work was partly supported by NSF grant OCE7727001 and the Deep Sea Drilling Project.

Received 22 February; accepted 23 May 1980.

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Deep water formation in the North Atlantic Ocean during the last ice age

Jean-Claude Duplessy

Centre des Faibles Radioactivités, Laboratoire Mixte CNRS-CEA, 91190-Gif-sur-Yvette, France

J. Moyes & C. Pujol

Institut de Géologie du Bassin d'Aquitaine, Université de Bordeaux I, 33405-Talence, France

Oxygen-18 records of benthic foraminifera from northeastern Atlantic and Southern Ocean cores are significantly different. This difference indicates that the deep water in the northeastern Atlantic Ocean during the last Ice Age was at least 1.3 °C cooler than in modern times. We show here that the occurrence of such a cold deep water mass implies that the North Atlantic was a sink for dense surface waters, replacing the Norwegian Sea where ice cover and stratification prevented the formation of deep water.

Over the last few million years, the Earth's climate has alternated between ice ages and warmer intervals. Although there have been many quantitative studies of the response of the ocean-atmosphere-ice system to climatic changes, most of these have been concerned with the history of ocean surface waters, based on faunal analysis of fossil plankton from deep sea sediments¹. However, the formation and circulation of deep water are also affected by climatic changes. The present major source of North Atlantic Deep Water (NADW) is the Norwegian Sea, in which an inflow of warm saline surface water is progressively cooled during its transfer towards high latitudes. This loss of heat to the atmosphere within the Norwegian basin increases the density of the surface water so that it sinks to the bottom, where it overflows the sills in the Denmark strait and the Faeroe channel and moves southward into the Atlantic Ocean². During the last ice age, however, the Norwegian Sea bottom water has been shown to be warmer than at present³, so that the Norwegian Sea was not a sink for surface water, which followed from the disappearance of the Norwegian current⁴ and stratification due to a permanent ice cover⁵. This implies a major change in the origin and circulation of the deep water of the Atlantic Ocean. Additional evidence for these changes is found in the benthic foraminiferal fauna changes of the North Atlantic Ocean during the last climatic cycle⁶⁻⁸; however, it has not been possible to interpret these faunal changes in terms of temperature variations of the NADW.

During the cruise FAEGAS 1 of the French RV Jean Charcot in the northeastern Atlantic, piston core CH 73139C was

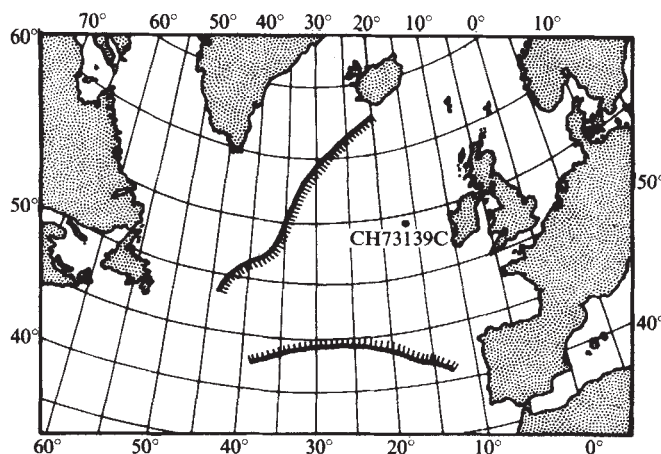


Fig. 1 Location of core CH 73139C. Lines with hachures indicate the extreme poleward and equatorward position of the polar front during isotopic stages 2, 3, 4. Core CH 73139C was north of the polar front during about half of this period¹⁸.

collected at 54°38' N, 16°21' W from a water depth of 2,209 m (Fig. 1). A sedimentological description of this core has been given elsewhere⁹. Radiocarbon dates have been obtained on the coarse fraction (>44 µm) which consists of a foraminiferal ooze with non-calcareous detrital material below 1 m. The mean sedimentation rate during the past 17,000 yr was calculated to be ~11.9 cm kyr⁻¹ (Table 1).

We have measured the ¹⁸O/¹⁶O ratio of the benthic foraminiferal species *Cibicides wuellerstorfi* and a mixture of *C. kullenbergi*, *C. gr. io* and *C. sp.* (ref. 10). Analyses were performed using a Micromass 602 C mass spectrometer with experimental procedures described elsewhere¹¹. Replicate analyses of the same carbonate gave a standard deviation of 0.07 ‰ for a single measurement. Results are expressed as δ¹⁸O defined by the relationship:

$$\delta^{18}\text{O} = \left[\frac{(^{18}\text{O}/^{16}\text{O})_{\text{sample}}}{(^{18}\text{O}/^{16}\text{O})_{\text{PDB}}} - 1 \right] \times 10^3$$

where PDB (Pee Dee Belemnite) is the Chicago PDB 1 standard.

Although most benthic foraminifera yield different oxygen isotopic values¹², all the *Cibicides* species from the same level in the sediment have identical oxygen isotopic ratios within experimental errors. Mean values for each level are reported in Table 2.

The isotopic record of benthic foraminifera in core CH 73139C is reported in Fig. 2a with a constant correction factor of 0.64 ‰ to take account of *Cibicides* departure from isotopic equilibrium^{8,13}. This record is readily correlated with the standard sequence of stages to stage 6 as defined by Emiliani¹⁴. The absolute δ¹⁸O values obtained and their variations with core depth closely match those from other cores from the North Atlantic Ocean^{8,15}, in agreement with the hypothesis that the major signal in these records reflects isotopic changes in the world's ocean water resulting from the variation of continental ice sheets.

The isotopic record shows that the sedimentation rate has varied during the last climatic cycle: assuming an age of 127,000 yr for the boundary between isotopic stage 5 and stage 6 (ref. 13), a mean sedimentation rate of 5 cm kyr⁻¹ is obtained below 1.8 m, which is about half of that determined for the upper part of the core. However, the sedimentation rate did not remain constant during that time. From the late glacial maximum to the boundary between isotopic stage 4 and stage 5, a mean sedimentation rate of 5.9 cm kyr⁻¹ can be calculated according to the time scale developed by Broecker and Van Donk¹⁶. Within stage 5, the fine structure of the isotopic record

Table 1 ¹⁴C dates of cores CH 73139C and MD 73025

Core CH 73139C		Core MD 73025	
Depth (cm)	Age (yr)	Depth (cm)	Age (yr)
7-13	2,020 ± 200	35-39	6,880 ± 330
46-50	5,300 ± 260	76-80	8,230 ± 370
77-83	8,520 ± 350	152-159	8,950 ± 400
112-120	12,020 ± 480	182-186	9,930 ± 440
140-144	11,800 ± 480	226-230	10,400 ± 460
157-165	15,230 ± 770	276-280	12,620 ± 550
180-184	16,900 ± 900	324-329	17,700 ± 1,000
220-224	23,300 ± 2,100	355-359	19,000 ± 1,200
		416-420	24,700 ± 2,400

A ¹⁴C half life of 5,730 yr has been used. No correction has been made to take into account isotopic fractionation in samples or apparent age of seawater carbon.

recognized first by Emiliani¹⁷ is not in evidence in core CH 73139C. However, the $\delta^{18}\text{O}$ peak between 720 and 620 cm can be unambiguously correlated with isotopic substage 5e on the basis of $\delta^{18}\text{O}$ values similar to the modern ones and of the occurrence in the planktonic foraminiferal fauna of warm species like *G. crassaformis* which has been reported only during that substage in the north-east Atlantic ocean¹⁸. Assuming an age of 115,000 yr for the boundary between isotopic substage 5e and substage 5d, a mean sedimentation rate of 8.3 cm kyr⁻¹ is calculated during substage 5e. In contrast, the sedimentation rate has dropped sharply between 115,000 and 75,000 yr BP (isotopic substages 5a to 5d) because only 1 m of sediment was deposited during that time. This low sedimentation rate (2.5 cm kyr⁻¹) is probably responsible for the blurring of the fine structure of the isotopic record¹⁴. Therefore, this part of the record will not be discussed in terms of palaeoceanographic change and we shall limit our discussion to the last 75,000 yr.

The present bottom water at the location of core CH 73139C is the North Atlantic Deep Water ($T = 3.3^\circ\text{C}$; $S = 34.96$); its $\delta^{18}\text{O}$ is +0.12 versus SMOW¹⁹. The measured $\delta^{18}\text{O}$ value for

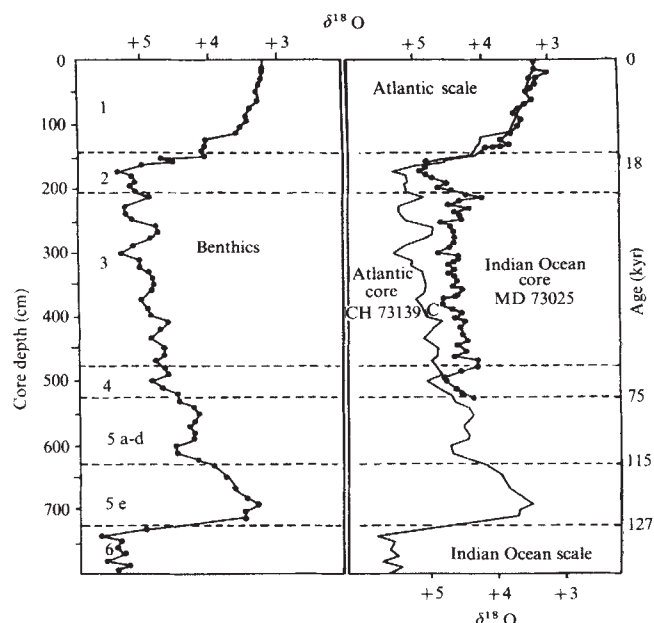


Fig. 2 a, Variations of $\delta^{18}\text{O}$ of *Cibicides wuellerstorfi* and of a mixture of *C. kullenbergi*, *C. gr. io*, and *C. sp. b*, Variations of $\delta^{18}\text{O}$ of benthic foraminifera in North Atlantic core CH 73139C and southern Indian core MD 73025 with time during the past 75,000 yr. A constant correction factor of 0.64 ‰ for *Cibicides* in core CH 73139C and of 0.40 ‰ for *Nonion* in core MD 73025 have been applied to take account of departure from isotopic equilibrium^{11,13,15}. Major isotopic stage boundaries¹⁴ are correlated with dashed lines.

Cibicides in the upper 10 cm of core CH 73139C is +2.58 versus PDB. Using a correction factor of 0.64 to take account of *Cibicides* departure from isotopic equilibrium^{8,13}, we calculated an isotopic temperature of $+3.5 \pm 0.4^\circ\text{C}$, again in good agreement with the modern conditions. The upper part of the benthic record contains $\delta^{18}\text{O}$ variations of high amplitude, up to 1.9 ‰ between the Holocene and the last glacial maximum. Such large $\delta^{18}\text{O}$ changes have also been reported for two other deep sea cores from the North Atlantic Ocean^{8,15}. These large variations reflect the fact that deep water in this area experienced large changes in physical parameters during the last climatic cycle.

In contrast, benthic records from the Pacific and Indian Oceans contain $\delta^{18}\text{O}$ variations of much smaller amplitude. One of the most detailed benthic records from this area is that of core MD 73025, collected in the diatom ooze belt at $43^\circ 49'\text{S}$, $51^\circ 19'\text{E}$ in a water depth of 3,284 m during the cruise OSIRIS I of the French MS Marion Dufresne in the Indian Ocean. Radiocarbon dates (Table 1) have been obtained on the total carbon content of the sediment and indicate that the most recent sediment has been lost during the coring process. However, the first 2.5 m of the core are Holocene in age and the sedimentation rate is as high as 54.5 cm kyr⁻¹. Between 11,000 and 24,000 yr BP, the sedimentation rate was only 11.7 cm kyr⁻¹ indicating a sharp decrease of the diatom production during the last Ice Age.

Digital $\delta^{18}\text{O}$ values for *Nonion* were reported by Duplessy¹¹. The isotopic record of core MD 73025 extends to Emiliani's isotopic stage 6, and, due to the high sedimentation rate, provides one of the most detailed isotopic sequence covering the last climatic cycle. The present bottom water at the coring site is the circumpolar water ($T = +0.6^\circ\text{C}$; $S = 34.69$). Using the measured $\delta^{18}\text{O}$ values for *Nonion* in the upper 10 cm of core MD 73025, using a constant correction factor of +0.4 ‰ to compensate for *Nonion* departure from isotopic equilibrium¹⁵, and using Shackleton's palaeotemperature scale which is valid for low temperature²⁰ when a $\delta^{18}\text{O}$ value of -0.3 versus SMOW is assumed for the Circumpolar Water¹⁹, we calculated an

Table 2 Oxygen isotopic analyses ($\delta^{18}\text{O}/\text{PDB}$) of *Cibicides wuellerstorfi* and *Cibicides* sp.*

Levels	$\delta^{18}\text{O}$ (<i>Cibicides</i>) <i>wuellerstorfi</i>	Levels	$\delta^{18}\text{O}$ (<i>Cibicides</i>) sp.
TOP	+2.59	340	+4.21
		350	+4.19
		360	+4.22
15	+2.58	370	
20	+2.57	375	+4.37
30	+2.60	380	
40	+2.64	390	+4.25
50	+2.67	400	+4.25
60	+2.66	410	+3.95
70	+2.75	420	+4.11
85	+2.82	435	+4.24
90	+2.83		
100	+2.89	450	+4.03
110	+2.93	460	+4.03
120	+3.40	470	+4.15
130	+3.41	480	+4.03
135	+3.46	490	+3.97
145	+3.42	500	+4.22
150	+4.06	510	+4.06
155	+3.88	520	+3.84
160	+4.34	530	+3.80
170	+4.73	540	+3.59
175	+4.51	550	+3.52
185	+4.46	560	+3.59
190	+4.53	570	+3.66
200	+4.48	580	+3.58
210	+4.22	590	+3.59
		600	+3.87
225	+4.62	610	+3.83
		620	+3.53
235	+4.61	630	+3.30
		645	+3.11
245	+4.50	665	+3.00
		680	+2.82
255	+4.14	690	+2.65
		700	+2.85
265	+4.10	710	+2.85
		720	+3.57
275	+4.22	730	+4.28
		740	+5.00
285	+4.49	750	+4.67
		760	+4.75
300	+4.68	770	+4.64
310	+4.39	780	+4.90
320	+4.41	790	+4.58
330	+4.25	795	+4.75

* Mean values are reported for each level.

isotopic temperature of $0.8 \pm 0.4^\circ\text{C}$, in good agreement with the modern temperature at the site. In this record in which both modern and last glacial maximum conditions are well documented, the amplitude of the isotopic shift between isotopic stages 2 and 1 is only 1.6‰.

The isotopic record of core MD 73025 is readily correlated with that of the high sedimentation rate core V19 29 from the equatorial Pacific Ocean, analysed by Shackleton²¹. We therefore conclude that the benthic records from the Pacific and Indian Oceans show highly correlated variations with an amplitude of 1.6‰ which is 0.3‰ smaller than that of the northeastern Atlantic cores.

Figure 2b compares the $\delta^{18}\text{O}$ records of the southern Indian Ocean core MD 73025 (ref. 11) and the northeastern Atlantic core CH 73139C during the past 75,000 yr. Both cores are plotted with the same time scale, but the $\delta^{18}\text{O}$ of the Indian Ocean record has been shifted by 0.25‰ to compensate for the present-day difference between the two sites. As the same species has been continuously analysed along each core, this shift, which brings into coincidence the two Holocene mean $\delta^{18}\text{O}$ values, allows us to compare the isotopic records at the two sites independently of the correction factors introduced to take into account the departure from isotopic equilibrium in benthic foraminiferal species. The two records are clearly significantly different and cannot both simultaneously reflect only continental ice volume variations. This amplitude difference cannot be explained through a smoothing of the Indian Ocean record due to benthic mixing as this core has a very high sedimentation rate: using the mixing model of Peng *et al.*²², ^{14}C data from core CH 73139C indicate an upper limit for the mixing layer thickness of 15 cm. This thickness, which is similar to that measured in most cores, is equivalent to a time interval of 1,400–3,000 yr, which is smaller than the duration of the full glacial conditions or any other climatic extreme. In fact, for both cores, the $\delta^{18}\text{O}$ values of glacial and interglacial extremes are well defined and practically unbiased by bioturbation. As North Atlantic Deep Water originates in the high latitudes of the North Atlantic Ocean whereas the circumpolar water originates mainly in the Weddell Sea²⁵ the differences between these two kinds of isotopic records indicate that the northern and southern sources of oceanic deep water have had different histories during global climatic variations.

The only explanation for such a difference between the isotopic records is a cooling of the northeastern Atlantic deep water: at present, Antarctic Bottom Water is formed during the winter when sea water freezes. As the ice forms, it rejects most of the salt from the water, so that the salinity and density of the water increase. As a result, this denser water sinks²³. As increasing bottom water formation during glacial periods has been inferred from particle size analysis of sediments of the Vema Channel²⁴, this enhanced formation process which occurs at the freezing point cannot produce bottom water warmer than that produced at present. Thus, as a conservative estimate, we shall assume that the deep water from the southern Indian Ocean experienced no temperature change in the past and that the $\delta^{18}\text{O}$ record of core MD 73025 reflects the normal glacial effect, which is continental ice volume variations. Many hypotheses can be advanced to explain the higher amplitude of the benthic record of core CH 73139C. For instance, one might argue that benthic foraminifera were heavy because the glacial NADW became isotopically heavier than expected from the normal glacial effect, without any temperature change. This hypothesis, however, is in conflict with all the reconstructions of the glacial North Atlantic^{1,25,26}. The surface salinity map for 18,000 yr BP clearly shows the occurrence of a well defined polar front at 42°N latitude, north of which the salinity decreased²⁶. The $\delta^{18}\text{O}$ of the surface water should also decrease in the same way. Palaeoceanographic reconstructions clearly demonstrate that there are ways (Labrador current, glacial flow) to get high latitude melt water to intermediate latitudes, north of the polar front. Thus there would be no way that deep water forming in this area could become enriched in ^{18}O , and the 0.3‰

amplitude difference between the Atlantic and south Indian benthic records indicates that the glacial NADW was at least 1.3°C cooler than at present.

A second hypothesis, agreeing with the occurrence of cold deep water, is that there was a complete absence of deep water formation in the North Atlantic during most of the last Ice Age, as suggested by the abundance of *U. peregrina*, a species living today in water with an oxygen content falling to below 5 ml per l (ref. 8). In such a case, the Southern Ocean would be the only source of the world ocean deep water. However, we can rule out this possibility as the benthic foraminifera from the North Atlantic Ocean were isotopically heavier than those of the southern Indian Ocean and Pacific Ocean²¹ during isotopic stages 2 to 4. If the only deep water source were located in the southern Indian Ocean, all the deep waters should have the same oxygen isotopic composition and our data would indicate that the glacial NADW was cooler than the deep water source itself, which is impossible.

We thus conclude that the North Atlantic Ocean has been a source of deep water during the past 75,000 yr, even during the coolest periods. The benthic foraminiferal data of Streeter and Shackleton⁸ clearly indicate that the dissolved oxygen content of this glacial NADW was sometimes low, reflecting large changes in the production rate of the NADW. During the period 15,000–75,000 yr BP, the glacial NADW was cooler than today. Our evidence is in direct conflict with the theories of Weyl²⁷ and Newell²⁸, who in their models of glacial ocean hypothesize warmer deep waters. In contrast, our results suggest that winter freezing in near shore areas, producing cold waters, is the most likely explanation of the isotopic composition of the benthic foraminifera in the North Atlantic Ocean during the last Ice Age. More cores will be necessary to determine the coolest deep water areas in the North Atlantic Ocean during the last climatic cycle, and thus the sources of the Glacial NADW.

As far as isotope stratigraphy is concerned, our data show that the major signal in the isotopic curves is an ice volume record but that minor fluctuations reflecting deep water temperature changes are superimposed on it. Until these temperature changes are more accurately known, worldwide correlations between isotopic records must be restricted only to the major trends which define Emiliani's stages.

We thank M. Kritz, H. M. Sachs, N. J. Shackleton and S. Streeter for reviews, J. Labeyrie for useful discussions B. Le Coat and J. Antignac for help in the isotopic analyses and A. Pujos-Lamy for the determination of the benthic foraminifera. The computer treatment of the data was made by D. Norde-mann. Carbon-14 measurements were made by G. Delibrias. We acknowledge the support of our study by CEA, CNRS and DGRST, the support of cruise FAEGAS of the French RV Jean Charcot by CNEXO and the support of cruise OSIRIS I by TAAF.

Received 13 March; accepted 16 May 1980.

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Enrichment of dissolved phenolic material in the surface microlayer of coastal waters

David J. Carlson & Lawrence M. Mayer

Department of Oceanography, University of Maine at Orono, Walpole, Maine 04573

The sea surface microlayer often has greater concentrations of dissolved organic material (DOM) than subsurface water^{1–5}, but the chemical nature of this material remains unresolved. Most studies of microlayer organic chemistry have focused on lipid^{6–9} and hydrocarbon components^{10,11}. Carbohydrate material can account for a significant fraction of the microlayer DOM⁴ supporting IR spectroscopic evidence which suggests that the microlayer material is predominantly carbohydrate-proteinaceous complexes¹². Some similarity of the microlayer DOM to marine humic material has been noted^{12–14}. We report here preferential enrichment of dissolved phenolic material in the surface microlayer of coastal waters.

Two major sources of phenolic substances in coastal marine waters are known. Terrestrial drainage delivers dissolved fulvic and humic material containing aromatic structures, partially phenolic, as important components^{15,16}. Marine macroalgae, especially the Phaeophyta, have been shown to exude significant quantities of dissolved polyphenols¹⁷.

The UV absorbance of seawater has been used to examine the distribution of DOM in marine waters¹⁸. Coastal waters exhibit UV absorbance in an inverse relationship to salinity, indicating a terrestrial origin for the substances causing absorption^{18–21}. Brown²² and Mrkva²³ have suggested that this riverine UV absorbance is due to aromatic compounds. The UV absorbance of macroalgal exudates has been ascribed to their polyphenol content¹⁷. Although UV absorbance has been shown to be inappropriate as an estimate of total DOM in coastal waters^{24,25}, it may be a useful parameter by which to monitor phenolic substances.

We have examined UV absorbance, dissolved organic carbon (DOC), and phenolic material in the bulk and surface microlayer waters of the Damariscotta and Saco River estuaries. The Damariscotta River estuary is a 30-km long, narrow embayment in central Maine with limited freshwater input, predominantly overflow from Damariscotta Lake. The Saco River is a major drainage in southern Maine with an 8-km long estuary. Microlayer samples were collected by immersing a 30×30 cm glass plate and withdrawing it vertically through the water surface²⁵. The glass plate collects a surface layer of water 51±2 µm ($\bar{x}$ ±95% C.I.) thick. All samples were filtered through pre-combusted, rinsed glass fibre filters, and the absorbance at 280 nm measured in 10 cm quartz cells. DOC was measured by persulphate oxidation and FR detection²⁷ using an Oceanography International carbon analyser. Dissolved phenolic material was analysed by a modification of the Folin-Ciocalteu (F-C) test²⁸.

The UV absorption of the estuarine waters varies almost linearly with their phenolic reactivity (Fig. 1), supporting earlier

suggestions that the UV absorbance of seawater reflects aromatic components. It is not possible definitively to identify the source of the phenolic material. Experiments with natural exudates from *in situ* macroalgae (*Ascophyllum nodosum* and *Fucus vesiculosus*) show that exudates have higher ratios of F-C reactivity to UV absorbance than do the lake, riverine and estuarine samples. However, the F-C reactivity, the UV absorbance, and the resultant reactivity to absorbance ratios of the exudates change with time (range indicated in Fig. 1), as does the UV absorbance of ethanol extracts of the same species²⁵, and this variation therefore causes some uncertainty as to the source of the estuarine phenolic materials. Bulk water and microlayer samples collected within the Damariscotta estuary show the same ratio of phenolic reactivity to ultraviolet absorbance as samples from Damariscotta Lake. Data from the Saco estuary, which has very little macroalgal input, show exactly the same trend as the Damariscotta estuary, demonstrating similarity between estuarine and lake- or river-derived phenolic material. Based on these data it seems that the surface microlayers of this estuary contain terrestrially derived phenolic material.

Data from the Damariscotta and Saco estuaries (Fig. 2) demonstrate consistent microlayer enrichment of UV absorbance, and hence phenolic material. The DOC levels, however, do not exhibit consistent enrichment. In addition, most samples plot above the line of equal microlayer partitioning of UV absorbance and DOC. Preferential enrichment of phenolic material relative to other DOC is therefore indicated.

Enrichment factors for UV absorbing material are plotted against salinity in Fig. 3. Enrichments of phenolic material occur at all salinities, but are demonstrably greater at higher salinities.

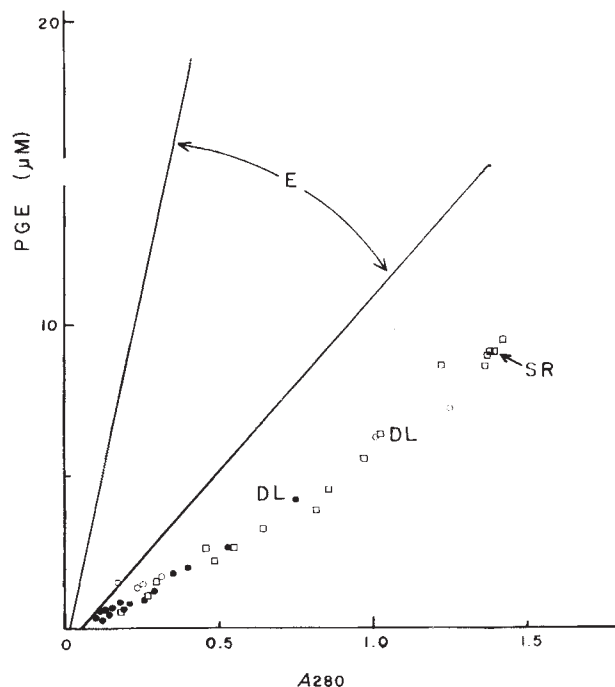


Fig. 1 Absorbance at 280 nm plotted against phenolic reactivity expressed as micromoles of phloroglucinol equivalent (PGE). Lower trend contains samples from Damariscotta transect of October 23, 24 and bulk water samples from the Saco R. estuary on 22 August, 1979. Macroalgal exudates (upper range) obtained by placing emerged fronds of *Fucus vesiculosus* and *Ascophyllum nodosum* in flasks of seawater before immersion by a rising tide. Data represent the difference between pre- and post-exudation values of PGE and A_{280} of the seawater in the flasks. Aliquots of the exudates were analysed after various time intervals. ●, Damariscotta bulk water; □, Saco bulk water; ○, Damariscotta surface microlayer; DL, Damariscotta Lake; SR, Saco River; E, algal exudates.

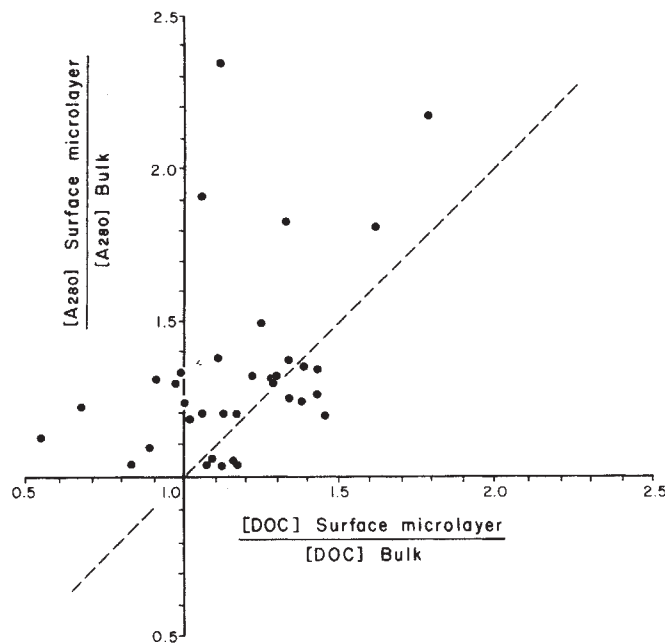


Fig. 2 Surface microlayer to bulk water ratios of DOC and UV absorbing material (A_{280}) in summer and fall 1979 samples from the Damariscotta River estuary and spring 1980 samples from the Saco River estuary. Only data without significant salinity differences between bulk and microlayer water are included. Dashed line indicates equal microlayer partitioning of DOC and UV absorbance. DOC values were $2.5\text{--}9.8\text{ mg l}^{-1}$ in bulk water and $2.6\text{--}11.9\text{ mg l}^{-1}$ in the surface microlayer.

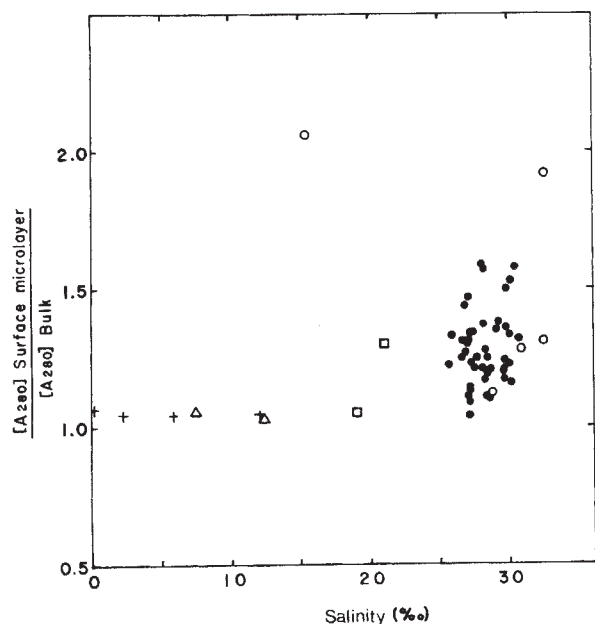


Fig. 3 Microlayer enrichments of UV absorbance plotted against salinity in samples from the Damariscotta and Saco estuarine systems. Data in which salinity differences between microlayer and bulk water exist have been included by correcting the bulk water absorbance to the absorbance appropriate at the microlayer salinity. ●, Damariscotta summer, 1979; △, Damariscotta autumn, 1979; □, Damariscotta spring 1980; +, Saco spring 1980.

We suggest three alternative explanations for this trend. First, Sholkovitz²⁹ has shown that terrigenous humic DOC becomes less soluble at high salinities. Higher partition coefficients at higher salinities can be explained if the dissolved phenolic material has similar chemical properties or if the enriched phenolic compounds are humates. Second, the rate of partitioning into the microlayer is unknown at this time; if these enrichments form slowly relative to the flushing time of terrigenous phenols through an estuary, then the higher enrichments at high salinities may simply reflect an ageing phenomenon. Third, if the UV absorbance at high salinities represents macroalgal rather than terrigenous phenolic material, we may be observing different partition coefficients for the two sources of phenols.

These strongly UV-absorbing molecules will be very susceptible to photo-oxidative transformations in the surface microlayer. This zone may therefore be a temporary sink and a reaction site leading to their resolubilization in bulk water as small degradation products³⁰ or to oxidation³¹ and removal to the atmosphere. These aromatic compounds will also serve as important sources of hydrated electrons³² or free radicals³¹ in the microlayer.

Sea surface microlayers often contain greater numbers of bacteria than subsurface water^{4,33–35}, and have been suggested to be heterotrophically active environments^{4,36}. However, apparent inhibition of bacterial activity in the microlayer has been reported^{4,37}, most notably in coastal waters^{35,38}. The presence of phenolic material, potentially inhibitory to bacteria^{36,39} and phytoplankton⁴⁰, in the surface microlayers of coastal waters may account for this reduction in microbial activity.

Funding for this study was provided by a National Wildlife Federation fellowship to D.J.C., the Faculty Research Fund of the University of Maine at Orono, and NSF grant OCE 79 20244. We thank the graduate students in the Department of Oceanography who assisted in the field work, and P. Rossi for her assistance with the graphics. The comments of W.B. Lyons and B. J. McAlice are gratefully acknowledged.

Received 22 January; accepted 29 May 1980.

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Late Jurassic lizards from Como Bluff, Wyoming and their palaeobiogeographic significance

Donald R. Prothero

Department of Vertebrate Paleontology, American Museum of Natural History, New York, New York 10024

Richard Estes

Department of Zoology, San Diego State University, San Diego, California 92182

Fossils from the late Jurassic Morrison Formation, Como Bluff, Wyoming (Tithonian, ~140 Myr ago) show that three genera of European lizards from the later Jurassic of England and Portugal (*Cteniohenys*, *Paramacellodus*, *Dorsetisaurus*) also inhabited North America. These specimens corroborate evidence from mammal and other terrestrial vertebrate faunal similarities that western Europe and western North America had a continuous land connection in the late Jurassic. Some of this similarity, however, is pre-Tithonian, indicating that widespread mid-Jurassic transgressive seas in Europe were no barrier to vertebrate dispersal.

Between 1968 and 1970, a joint American Museum of Natural History–Yale University expedition made new collections at Quarry Nine, the famous 'mammal quarry', in the Upper Jurassic Morrison Formation at Como Bluff, Wyoming. The expedition collected many mammalian specimens described elsewhere¹, as well as dinosaur material, glyptodont turtles², crocodiles, sphenodontids, frogs, lungfish, amiod fish and lizards. A review of this and previously known lizard material from the Morrison Formation has resulted in the identification of at least three genera of lizards. Only one, *Cteniohenys antiquus* Gilmore 1928, was known previously from North America; it has been shown to be an eolacertilian³.

Hecht and Estes⁴ reported an ilium of what they believed to be a true lacertilian from previous Quarry Nine collections, but did not name it. Lacertilian ('true') lizards are known from the late Jurassic of Europe and China⁵. A presumed lacertilian was described from the late Triassic of China⁶, but one of us (R.E.) believes it to be an eolacertilian. The new material from the Morrison Formation not only verifies the occurrence of both eolacertilians and lacertilians in the late Jurassic of North America, but also shows for the latter group a well-established, derived, 'autarchoglossan' organization at this early time, which is in agreement with previous identifications of some Jurassic European forms as scincomorphs. The three lizard species identified in the Morrison Formation are classified as follows:

Order Squamata
Suborder Lacertilia
Infraorder Eolacertilia
Cteniohenys antiquus Gilmore 1928

The new collection includes two jaws referable to this species. (Other jaws, including the type specimen, were found in the original Marsh collections made at Como Bluff in 1879 and 1880.) *Cteniohenys* was later identified by Seiffert³ from the late Jurassic (Kimmeridgian) fauna from Guimarota, Portugal. We agree with Seiffert's identification of *Cteniohenys* as an eolacertilian, closely related to the 'flying lizards', or kuehneosaurs, known from the late Triassic of Europe⁷ and North America⁸. *Cteniohenys* is known from jaws, maxillae and parietals. The other species are true lacertilians, of which the first is:

Infraorder Lacertilia
Paramacellodus sp.

Paramacellodus oweni was first described from the late Jurassic Purbeck Formation of England, and allied with the cordyloid

scincomorphs⁵. Several jaws referable to *Paramacellodus* (including two from the new collection) occur at Quarry Nine (Fig. 1a,b); they may represent the same species but the material is insufficient to determine this. Some postcranial material possibly referable to *Paramacellodus* also occurs in the new collection. The specimen (AMNH 11523) consists of a left prefrontal, a left scapulocoracoid, one trunk vertebra, a partial pelvis, seven caudal vertebrae, and a nearly complete left hind limb. The hind limb, pelvis and sacral vertebrae are articulated. All other elements are disarticulated, but closely associated on the block of matrix.

A detailed description of this material is in preparation, but the important features of AMNH 11523 can be summarized as follows. The prefrontal has a sculpture pattern like that of an unidentified Jurassic specimen from Portugal (Fig. 1c,d)³ and may possibly represent the prefrontal of *Paramacellodus*. The scapulocoracoid (Fig. 1e,f) is fully lacertilian, with expanded coracoid region that resembles the derived condition in cordyloid lizards. In the pubis, the iliac blade differs in shape from the ilium described by Hecht and Estes⁴, but both are generally similar in morphology to the ilia of some cordyloid lizards (Fig. 1g-j). In the tail (Fig. 1k), the transverse process of the sixth vertebra may show an autotomic septum, implying fragility of the tail. If so, the septum would split the vertebra into two parts as in many lizards. Comparison of AMNH 11523 with recent lizard skeletons showed that this autotomic configuration is very similar to that of comparably sized lizards belonging to several families, particularly some scincids; cordyloids have the autotomic plane anterior to the transverse process. The third species is:

Family Dorsetisauridae
Dorsetisaurus sp.

There are two dentaries of *Dorsetisaurus* sp.⁵. Neither specimen is well preserved (Fig. 1l-n), and both are visible only in lateral view. The elongated, flattened, blade-like teeth, some of which are antero-posteriorly expanded, make this identification clear. Hoffstetter, who originally described *Dorsetisaurus*, placed it in its own family Dorsetisauridae, with which we agree. He also included the family in the Anguimorpha; if so, dorsetisaurus would be very primitive members of that group.

The presence of three genera of small lizards in both North America and Europe in the late Jurassic is an indication of the close faunal affinities of the two regions at that time. Continental reconstructions show that the North American plate was still in contact with Europe at the end of the late Jurassic^{11,12}. Como Bluff, Wyoming, and southern England were approximately 2,500 km apart, equivalent to the present distance between San Francisco, California and St Louis, Missouri. Some recent lizards have ranges larger than this. In addition, several other small animals apparently ranged from England to the present western United States. These include four genera of shrew-sized mammals^{1,13,14} (*Docodon*, *Amblotherium*, *Ctenacodon*, *Trioracodon*), the crocodile *Goniopholis*¹⁵, the dinosaur *Camptosaurus*¹⁶, and the amiod fish *Ophiopsis*¹⁷. Also, most of the Morrison mammal taxa have their closest sister-groups in Europe, and a cladogram of therian mammals shows no separation of the taxa into European and American clusters, as might be expected if long-term endemism had taken place¹.

Direct comparisons have not been made in other groups, but most other elements in the Morrison fauna have their closest relatives in the European later Jurassic. Turtles from the early Cretaceous of England¹⁸ and sphenodontids from the Kimmeridgian of Portugal¹⁹ have been examined recently, and are most closely related to Morrison taxa. Complete faunal lists of the Kimmeridgian Guimarota fauna of Portugal have not yet been published, but the partial one given by Kühne¹³ is very similar to that from the Purbeck and Morrison Formations. To date, the mammals^{13,14}, dinosaurs²⁰, crocodilians²¹, turtles²² and lizards³ have been described, and most forms show their closest

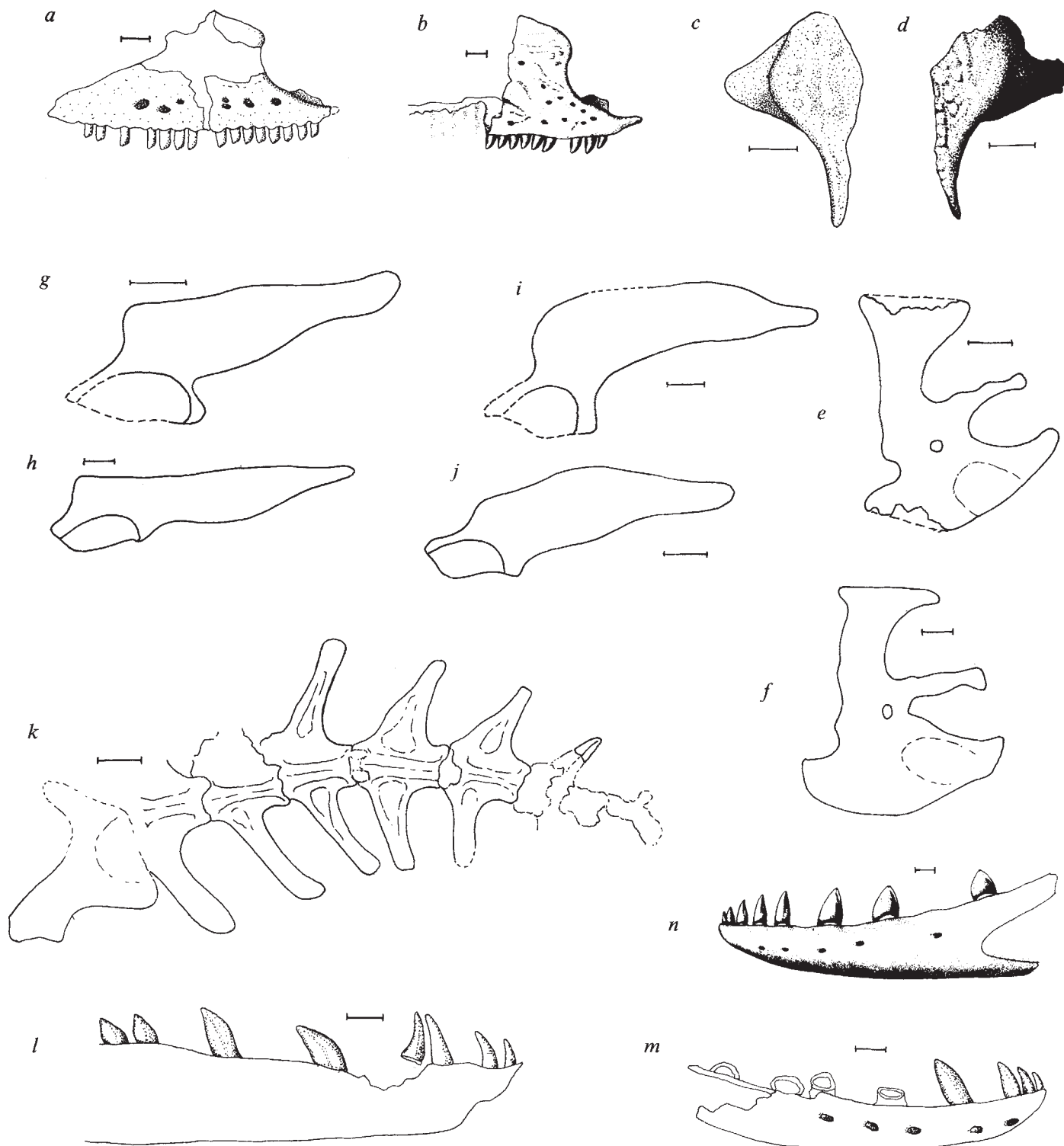


Fig. 1 *a*, *Paramacellodus* sp., right maxilla, Morrison Fm. *b*, *Paramacellodus oweni*, right maxilla, Purbeck Fm⁵. *c*, AMNH 11523, left prefrontal, Morrison Fm. *d*, GUI. 157, right prefrontal, Guimarota Beds³. *e*, AMNH 11523, left scapulocoracoid, Morrison Fm. *f*, Left scapulocoracoid of recent *Cordylus polyzonus* for comparison. *g*, AMNH 11523, left ilium, Morrison Fm, comparable with *h*, recent *Platysaurus guttatus*. *i*, YPM 1568, left ilium, Morrison Fm, comparable with *j*, recent *Cordylus warreni*. The fossil iliac blades show some differences, but are within the range of variation of modern cordyloid lizards. *k*, AMNH 11523, caudal vertebrae. *l-m*, *Dorsetisaurus* sp., dentary, Morrison Fm. Both specimens are badly broken. *n*, *Dorsetisaurus purbeckensis*, dentary, Purbeck Fm⁵. All scale bars, 1 mm.

affinities with forms from the Morrison and Purbeck Formations. Kimmeridgian sauropods from Portugal (*Apatosaurus*, *Brachiosaurus*) are congeneric with Morrison taxa²³. Pre-Kimmeridgian Jurassic dinosaur faunas in both North America and Europe are highly similar^{24–26}. Bathonian (middle Jurassic) mammals from England²⁷ and Scotland²⁸ show close affinities with Morrison mammals¹.

These strong faunal resemblances between western Europe and western North America seem to indicate a continuous land connection between these regions throughout the Jurassic. Yet reconstructions by Ager²⁹, Hallam³⁰, and Hallam and Sellwood³¹ show both southern England and Portugal submerged or surrounded by seaways during most of the Jurassic. The maximum extent of Jurassic epicontinental seas was during the Oxfordian and Kimmeridgian (early late Jurassic)³⁰. During the Tithonian, widespread tectonism uplifted large areas of land, reconnecting Europe and North America^{30,31}. The amount of exposed land in the late Tithonian reached a maximum level matched only by the earliest Jurassic (Sinemurian–Pliensbachian) regression³⁰.

If the similarity of land faunas were restricted to the late Tithonian, then rapid migration during the maximum phase of regression would be sufficient to explain the lack of endemism. But the similarity persists throughout the Jurassic, indicating that the submergence was not continuous or effective enough to be a barrier to vertebrate migration. Hallam (personal communication) has suggested that the epicontinental seas were so shallow that the numerous short-term regressions occurring throughout the Jurassic³⁰ would create transient land corridors. Migration then reached a peak during the Tithonian when dry land connection extended across Europe and North America.

We thank M. C. McKenna, E. S. Gaffney, R. J. Emry and J. H. Ostrom for permission to study specimens in their care. The new Como Bluff specimens were collected by T. H. Rich, prepared by C. S. Schaff and curated by E. Manning. We thank H. Green, A. Hallam, J. Gauthier and E. Manning for helpful comments. D.R.P. was supported by a Columbia Faculty Fellowship and an NSF graduate fellowship and R.E. was supported by NSF grant DEB-77-14995.

Received 22 February; accepted 9 May 1980.

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Green anole in Dominican amber

Olivier Rieppel

Department of Zoology, Natural History Museum, Basel, Switzerland

A lizard of the iguanid genus *Anolis* enclosed in amber of Oligocene or Miocene age from the Dominican Republic represents the first complete vertebrate fossil in amber as well as the oldest and only complete fossil of its genus. The fossil *Anolis* is very closely related to a recent form and will shed some light on the evolution of its genus which otherwise has a poor fossil record.

The evolution of the iguanid genus *Anolis* on the Caribbean islands serves as a model for the ecology of colonization and speciation on small islands^{1,2}. The evolution of the Caribbean *Anolis* has been reconstructed by Etheridge on the basis of osteology and by Gorman *et al.* on the basis of cytology. Their findings have been summarized by Williams¹. Etheridge was able to divide the genus into an α and a β section. α *Anolis* lacks transverse processes on the caudal vertebrae whereas they are present on β *Anolis*. A striking result of Etheridge's analysis is that the α *Anolis carolinensis* from the south-eastern United States is not related to the β anoles from Mexico, but it has Caribbean affinities, being related to the species of the *carolinensis* complex of the western Caribbean. *Anolis carolinensis* invaded the continent from a tropical island. The geographic centre of the *carolinensis* complex today is Cuba. Some slightly more primitive α anoles occur on Hispaniola. Of these, members of the 'green anole species group' as defined by Williams³, such as *A. chlorocyanus* or *A. coelestinus*, were found by Etheridge to be most closely related to and possibly ancestral to the *carolinensis* complex.

This phylogenetic scenario is usually assumed to have taken place during the Pleistocene. The genus *Anolis* is thought to have colonized the Caribbean islands after the Early Miocene inundation¹, but no fossil remains of these lizards older than the latest Pleistocene age have been reported, either from the Caribbean islands or from the southeastern United States. The oldest fossil *Anolis* remains are skin fragments enclosed in amber of Oligocene or Miocene age from Mexico⁴.

In 1979, Dr C. Baroni-Urbani from the Natural History Museum in Basel obtained a more or less completely preserved specimen of *Anolis* in amber from the Mina 'La Toca', Cordillera Septentrional, Dominican Republic (Fig. 1). Dominican amber is thought to be of middle Oligocene or early Miocene age⁵. The generic affinities of the fossil lizard are indicated by the structure of the toes, which bear scansorial pads on the lower surface of their expanded distal parts (Fig. 1). X-ray analysis revealed an incompletely preserved skeleton. Important characters such as the structure of the pectoral girdle or the number of presacral vertebrae cannot be determined. However, caudal vertebrae can be shown to lack transverse processes, a feature characteristic of α anoles (Fig. 2).

In view of its uniqueness I propose to name the fossil anole:

Anolis dominicanus n. sp.

Holotype: Natural History Museum Basel, Department of Entomology collection no. P 52. Mina 'La Toca', Cordillera Septentrional, Dominican Republic.

Diagnosis: A member of the α section of the genus *Anolis*: ventral scales hexagonal, subimbricate, smooth; scales on tail imbricate, keeled, arranged in verticils comprising 4–5 middorsal scales; 22–24 lamellae under fourth toe.

Description: The fossil *Anolis* has a total length of approximately 71 mm; the head is 9.2 mm long; snout–vent length is 26.5 mm; the tail is complete and not regenerated. The left front limb is difficult to measure because of three-dimensional

*Present address: Palaontologisches Institut und Museum der Universität, Künsterlegasse 16, 8006 Zürich, Switzerland.

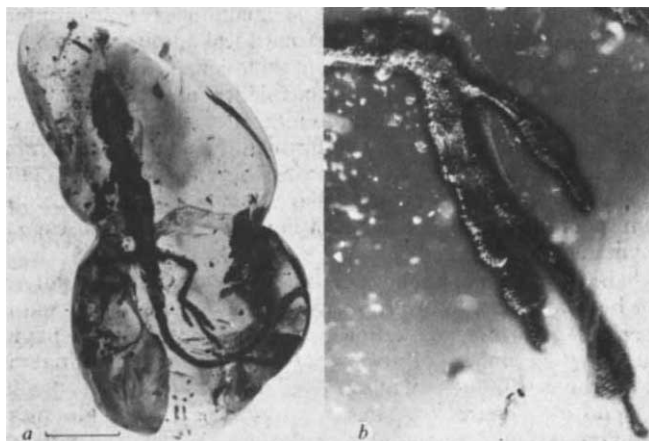


Fig. 1 *a*, *Anolis dominicanus* n. sp., holotype, as preserved in dorsal view. Scale bar, 10 mm. *b*, Lower surface of enlarged right pes, showing the lamellae on fourth digit.

distortion; it is approximately 12 mm long. The right hind limb can be measured accurately: its length up to the tip of the fourth toe is 17.3 mm. The total length of the specimen indicates either a juvenile or a small species.

The colour of the lizard has not been preserved. The squamation of the dorsum lies immediately below the surface of the amber and cannot be made out in detail. The scales on the ventrum are partially preserved. They are hexagonal, sometimes with a rounded posterior edge, subimbricate and smooth. The scales covering the gular region are only partially preserved. They are smaller than the ventral scales, granular, juxtaposed and smooth. Gular scales smaller than ventrals probably imply that the specimen is a female. A weakly expressed longitudinal gular fold is observed. The dewlap appears to have been small or vestigial, which might be correlated with the juvenility or with the sex of the specimen, or it might represent a primitive characteristic.

The tail is trigonal in cross-section. The scales of the tail are imbricate, keeled and arranged in verticils. The verticils are sometimes not distinctly set off from one another. They can well be made out in a middle section of the tail where I counted 5-4-5-4-4-4 middorsal scales in 6 verticils.

The lower surface of the expanded distal part of the fourth toe certainly bears more than 20 lamellae: the exact number is difficult to establish because of a break in the amber obscuring the view (Fig. 1). There could be 22-24 lamellae, 23 being a probable number.

As described above, all the visible characteristics of *A. dominicanus* match Williams's³ definition of the Hispaniolan 'green anole species group', a group of four rather generalized species of moderate to small size and green colour, which live in the crowns of trees. Within the 'green anole species group', the

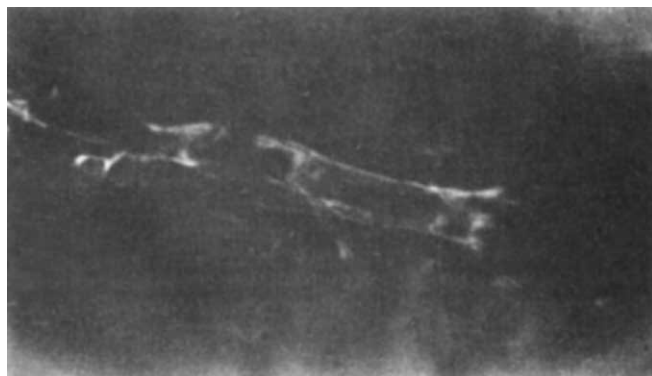


Fig. 2 X-ray picture of a well preserved vertebra from the posterior part of the tail, showing the absence of transverse processes. The length of the vertebra equals ~1 mm.

Table 1 Body measurements of Hispaniolan green anole species

Species	n	SVL (mm)	HDL = % SVL	HLB = % HDL
<i>A. chlorocyanus</i>	9	34.5-70	31 (32.7) 34.8	178.4 (190.6) 206.6
<i>A. coelestinus</i>	11	26-77	29.8 (34) 38.4	180 (190.9) 200
<i>A. aliniger</i>	10	41.8-60.5	29.5 (31.7) 35	151.6 (162.3) 186.2
<i>A. singularis</i>	17	37-73	29.8 (32.5) 35.4	157.1 (172.3) 187
<i>A. dominicanus</i>	1	26.5	34.7	188

n, Number of specimens examined; SVL, snout-vent length, the maxima and minima values of all specimens examined are indicated; HDL, head length, expressed as % of snout-vent length; HLB, hind limb, expressed as % of head length. The figures indicate maxima and minima, and the arithmetic mean of all specimens examined is given in parentheses. Measurements are based on the following specimens: *A. chlorocyanus cyagnosticus*, MBS 19778, 20100, 20268, 20344-45; *A. c. chlorocyanus*, MBS 19834, 19975, 20302-03; *A. coelestinus*, MBS 3056-58, 19841-43, 19853-54, 20402-03, 20405; *A. aliniger*, MCZ 143367-76; *A. singularis*, MCZ 124629-45. MBS, Natural History Museum Basel; MCZ, Museum of Comparative Zoology, Harvard University.

first subgroup (*A. chlorocyanus*-*A. coelestinus*) is characterized by somewhat longer hind limbs (190% of head length) than the second subgroup (*A. aliniger*-*A. singularis*, hind limbs 162-172% of head length). *A. dominicanus* shows a relative length of the hind limb as it is observed in the *A. chlorocyanus*-*A. coelestinus* subgroup (Table 1). Among the green anoles of Hispaniola, *A. coelestinus* is the species with the relatively longest head. The proportions of the head are the same in *A. dominicanus* (Table 1).

In conclusion, the fossil *A. dominicanus* from Dominican amber is evidence of the presence of α anoles closely related to recent forms (especially *A. coelestinus*) since the Oligocene or early Miocene. This is a much earlier date than that which has usually been assumed to account for the evolution and dispersal of α anoles on the Greater Antilles.

I thank Drs E. E. Williams and R. Etheridge for loaning specimens, critically examining X-ray pictures and reading a draft of this report. X-ray analysis was done by Professor W. Stürmer.

Note added in proof: New dating of amber matrix from the Dominican Republic by Dr J. B. Saunders, Basel, indicates an age range of between 20 and 23 million years BP (lower part of early Miocene).

Received 25 April; accepted 20 May 1980.

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Unique adaptations for submarine pollination in seagrasses

J. M. Pettitt*, C. A. McConchie, S. C. Duckert† & R. B. Knox†

Department of Botany, British Museum (Natural History), Cromwell Road, London SW7 5BD, UK

School of Botany, University of Melbourne, Parkville, Victoria, Australia 3052

Seagrasses are flowering plants that are adapted for pollination submerged in the sea. Many species, including the Australian sea nymph, *Amphibolis antarctica*, have filiform pollen grains. These grains have no exine¹⁻⁴—the resistant pollen wall layer characteristic of terrestrial plants—and each may be up to 5 mm in length. The pollen has a similar density to seawater so that it remains suspended or floats after release from the anther. The stigma of the sea nymph and related seagrasses is a simple branched structure which protrudes between the leaves when receptive and has a surface secretion with cytochemically detectable enzymatic activity^{1-3,5}. This stigma coating is not

dispersed in seawater. However, submarine pollen-stigma interactions have never been observed in any species of seagrass. We demonstrate here that pollination in the sea nymph has several unusual features when compared with the events in terrestrial flowering plants. These features include the waterproof nature of the adhesive binding the pollen to the stigma surface, the unique mechanism for pollen germination in the inaperturate filiform grains and the mode of pollen germination in the inaperturate filiform grains, and the mode of pollen tube penetration into the stigma. Taken together these characteristics suggest a considerably more refined level of adaptation to the marine environment than hitherto supposed.

Mature flowering plants of *Amphibolis antarctica* were collected from natural populations growing on the coast of Victoria, Australia, and held in aerated seawater aquaria at 14 or 22 °C. Ripe pollen from dehiscing anthers was taken up in a Pasteur pipette and applied to the stigmas in a stream of seawater. Grains adhered immediately on contact, and were allowed to remain for up to 48 h before fixation in buffered 2.5% glutaraldehyde and processing for light and electron microscopy⁷.

The pollen grains are held tenaciously on the stigma by a meniscus of adhesive material which is formed on contact from the surface coating of both interacting surfaces (Fig. 1a). This adhesive is waterproof, a quality which contrasts with the adhesive of terrestrial plants. In *Gladiolus*, for example, water-soluble arabinogalactan proteins have been implicated in pollen-stigma adhesion^{6,7}. Germinated pollen produced numerous tubes in the vicinity of the contact zone with the stigma (Fig. 1b). We have observed that the grains failed to germinate until they came into contact with the submerged stigma, suggesting that molecular transfer may be important for promoting pollen tube growth, as in the seagrass *Zostera marina*⁸.

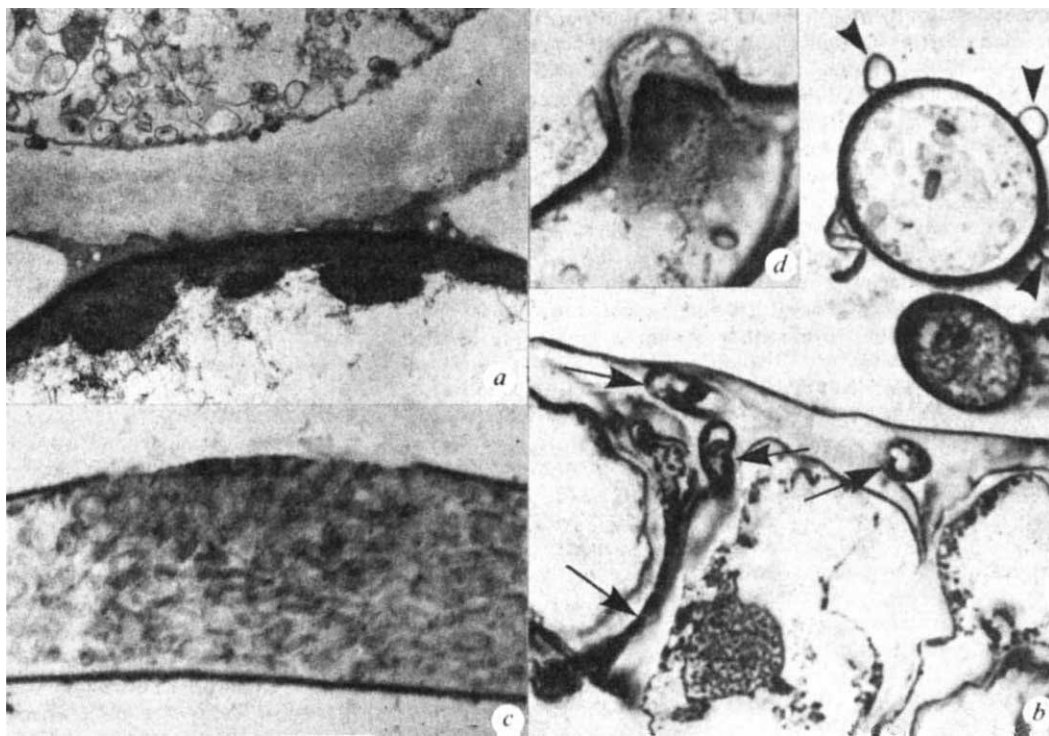
The filiform grains of seagrasses do not have preformed apertures—special sites for pollen tube emergence. Instead, the position of the aperture is first detectable cytologically by a marked loss of staining affinity in the wholly fibrillar wall⁴ after

attachment of the grain to the stigma (Fig. 1c). Our interpretation of this phenomenon is that focal autolysis forms the aperture. Reorganization of cytoplasmic components, including starch grains, is seen beneath the incipient aperture (Fig. 1c), but the first indication of pollen tube emergence is the appearance of a mucilaginous cap protruding through the aperture to form a bubble-like structure. This overlies the emerging tube cytoplasm (Fig. 1d) and the cap mucilage stains a light purple colour with toluidine blue, indicative of low molecular weight carbohydrates⁹.

The formation of pollen apertures by means of focal autolysis of the wall is not known for any other flowering plant, and is perhaps a further adaptation to the aquatic environment. In germinating pollen of terrestrial angiosperms the intine is contiguous with the pollen tube wall in both aperturate^{10,11} and inaperturate grains^{12,13}. Thus, our observations call into question the assumed homology of the seagrass pollen wall with the intine of their land-based relatives, notwithstanding the structural and cytochemical similarities²⁻⁵.

Hydration is an essential prelude to germination of the pollen of terrestrial angiosperms^{6,11,12,14,15}. In seagrass pollen, however, the hydrodynamic principles must be different, although our observations show that submarine germination is triggered by stigma contact. The differences extend also to subsequent reproductive events. In *Amphibolis*, the germinating tubes grow within a water-resistant mucilaginous sheath towards the stigma surface where they make contact with the cuticle. Using light microscopy, we have observed digestion of the cuticle by the pollen tubes and entry of the tubes into the polysaccharide matrix of the receptive cell wall (Fig. 1b) as well as initial growth through the transmitting tissue to the ovary. This sequence first necessitates penetration of the cuticle, a process which accommodates one level of control in those terrestrial angiosperms with dry stigma types, where cutinase activation is implicated^{6,12,14,16,17}. Precisely how these cutinases are produced and deployed in the seagrasses remains to be determined.

Fig. 1 *a*, A meniscus of insoluble adhesive forms at the point of contact between the pollen grain (upper half) and the stigma in *Amphibolis antarctica* and binds the two together. Electron micrograph of a section stained with silver proteinate after periodate oxidation²¹ $\times 14,400$. *b*, Pollen at the surface of the stigma. The filamentous grains have been sectioned transversely and the circular profiles of several pollen tubes passing towards the stigma are indicated by arrow heads. Pollen tubes are also present beneath the stigma cuticle in the walls of the receptive cells (arrows). Thick section of resin-embedded specimen stained with toluidine blue, $\times 1,200$. *c*, The aperture for pollen tube emergence is formed by autolysis of the pollen grain wall. The site of the future aperture is first detectable by the loss of staining affinity in the wall and reorganization of the cytoplasmic components beneath the site. Staining as for *b*, $\times 800$. *d*, After formation of the aperture pollen tube emergence begins. The first structure to appear is a mucilaginous cap and immediately behind this is a zone of densely staining cytoplasm. The pollen grain wall forming the border of the aperture flanks the emerging pollen tube. Staining as for *b*, $\times 960$.



The special adaptations to submarine pollination are important for an understanding of the mechanisms associated with discrimination of self (compatible) from foreign seagrass pollen in interspecific and intergeneric matings. Two species of *Amphibolis* are sympatric in southern Australian waters and other seagrass genera occupy adjacent habitats. Although the flowering periods are often coincidental, authenticated hybrids are unknown. In terrestrial pollination systems, barriers to discriminate against foreign pollen operate at initial contact, before adhesion, when many foreign grains simply fail to hydrate, while others are blocked at later steps leading to fertilization^{6,11,12,14,15}. Our observations of the seagrasses suggest that barriers to foreign pollination may operate at the moment of adhesion, or even at the time of stigma penetration within the waterproof coating. Experiments are now in progress to test the validity of these predictions.

It is generally believed that seagrasses are angiosperms which

have adapted successfully to their marine environment by the loss or modification of terrestrial features^{18,19}. In view of both the present discoveries and the distinctive structural and physiological features—such as filiform and flexible pollen grains up to 5 mm in length; precocious microspore mitosis following soon after tetrad period in pollen development^{3,20}; the wholly fibrillar nature of the pollen wall in many species¹⁻⁵; water-resistant surface-coating of the receptive stigma¹ which also possesses acid phosphatase activity⁵ as well as esterase activity characteristic of terrestrial stigmas; and coincidence of flowering in some species with tide cycles⁵—the explicit phylogenies of the groups need to be determined.

We thank the Australian Research Grants Committee for financial support (to S.C.D. and R.B.K.) and the Royal Society for the award of a Royal Society and Nuffield Foundation Bursary to enable J.M.P. to work at the University of Melbourne.

Received 31 March; accepted 21 May 1980.

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Relict diapause in an introduced weevil in New Zealand

S. L. Goldson

Ministry of Agriculture and Fisheries, PO Box 24, Lincoln, New Zealand

R. M. Emberson

Department of Entomology, Lincoln College, Canterbury, New Zealand

The Argentine stem weevil *Hyperodes bonariensis* Kuschel, a stem boring weevil native to the southern part of South America, was first reported in New Zealand in 1927¹ where it has become a widespread and serious pest of Graminae. Recent investigations² have demonstrated the existence of reproductive hibernatory diapause induced by a critical photoperiod in the population in Canterbury, New Zealand (latitude 43°39' S). Coincidentally, it was noted that similar ovarian diapause occurs in *H. bonariensis* found at a corresponding latitude (41° S) in the Bariloche region of Argentina³. Owing to the imminence of the autumnal equinox at diapause induction (early March in both localities), identical daylengths are experienced successively over a wide latitudinal range within a week. In the present report, examination of the New Zealand population at this time, sampled over at 8° range of latitude, revealed a corresponding synchronous induction of diapause without the usual latitudinal and therefore temperature-related shift in critical photoperiod⁴. This lack of temperature-related shift and the relatively mild winters suggest that the diapause behaviour of *H. bonariensis* throughout New Zealand is an adaptively inappropriate relict response resulting from the recently arrived weevils cueing into the same photoperiodic seasonality as their South American counterparts, which in regions such as Bariloche, must have evolved diapause behaviour to survive.

During a 3-day period, samples of weevils were collected from localities near sea level in the North Island of New Zealand between 35°39' S and 40°23' S and examined by dissection for diapause status. This allowed comparison with comprehensive data collected from Canterbury on *H. bonariensis* diapause induction during 1977–79.

The results, summarized in Table 1, show the similarity of diapause condition found in the populations sampled from four widely separated locations at the end of the second week of March. Surprisingly, the diapause-related physiological reorganization seemed somewhat further advanced in the populations collected from the warmer northern areas. This is indicated in Table 1 by the percentage of the population with clearly defined oocyte resorption bodies⁵. When a population is fully reproductive, oocyte resorption bodies are very rare and 80–90% of the reproductively competent weevils have eggs. Table 2 shows that during diapause induction (28 February–14 March) daylengths of increasing shortness were experienced in Whangarei (35°39' S) about a week before identical daylengths were experienced at Lincoln (43°39' S). This implies that the populations of weevils in the northern areas would have experienced the presumed common critical photoperiod shortly before their Lincoln counterparts and hence accounts for the slightly more advanced diapause condition of these northern populations.

Table 1 indicates that the time of diapause induction of Argentine stem weevil was completely unaffected by variation in mean daily temperature over a considerable latitudinal range. Such a response to photoperiod irrespective of ambient temperature is frequently observed in other species within a local region, as there is a stable correlation between the seasonal course of daylength and temperature. However, this correlation varies sharply with latitude. Daylength provides a reliable means of anticipating winter conditions independent of the vagaries of weather. In general, with an increase in latitude, the cooler conditions necessitate earlier onset of hibernatory diapause which is often regulated by longer critical photoperiods⁴. Thus, intraspecific latitude (and therefore temperature) related variation in critical photoperiod is to be expected in widely dispersed hibernating insect populations. In spite of this

Table 1 Reproductive status of female *H. bonariensis* at four localities in New Zealand

Locality	Date sampled	% With eggs	% With oocyte resorption bodies	% Fertilized	% Fertilized with eggs	No.
Whangarei	13.3.80	6.5	48.3	67.7	9.5	31
Auckland	14.3.80	6.5	36.7	51.6	12.5	31
Palmerston North	11.3.80	0	30.4	34.8	0	23
Lincoln	13.3.79	10	15	30	33.3	20
	12.3.78	5	25	45	11.1	20
	13.3.77	14.3	—	71.4	20	20

Table 2 Environmental factors at the sampling localities^a

Climatological station	Latitude	Daylength (hours and minutes)			Mean daily screen temperature (°C)					
		28 Feb.	7 March	14 March	March	April	May	June	July	August
A 54631 Glenbervie Forest Whangarei	35°39'S	12.52	12.38	12.21	16.5	14.7	12.3	10.2	9.0	9.7
C 64971 Mangere Auckland	36°58'S	12.57	12.42	12.25	17.8	15.8	13.2	11.0	10.2	10.8
E 05363 Palmerston North DSIR	40°23'S	13.04	12.46	12.27	16.1	13.7	10.7	8.4	7.8	8.9
H 32641 Lincoln	43°39'S	13.14	12.52	12.29	14.1	11.4	8.1	5.6	4.8	6.4

Daylength from nearest airport through NZ Civil Aviation Administration. Expressed as time from sunrise to sunset.

there was no evidence of a temperature-compensated shift in critical photoperiod among any of the *H. bonariensis* populations sampled (Table 2).

The results of this experiment confirm the exotic origin of the *H. bonariensis* population in New Zealand. With an estimated overall threshold temperature of development of ~10°C (ref. 6) there seems to be little adaptive reason for diapause during the comparatively mild New Zealand winters, particularly in the north. The seasonal behaviour of *H. bonariensis* in New Zealand is undoubtedly a relict response resulting from the insect cueing into a photoperiodic seasonality identical to that of its native habitat. In some South American localities the evolution of hibernatory diapause must have been necessary to allow the weevil to survive the relatively severe winter conditions, such as those of Bariloche (a ski resort) in Argentina. Here, at 41° S, *H. bonariensis* has been observed in diapause between March and July, as occurs in New Zealand.

The relict diapause behaviour of *H. bonariensis* in New Zealand therefore elegantly demonstrates the overriding effect of photoperiod on the adult stage resulting in diapause induction irrespective of other abiotic factors and the associated adaptive implications. In effect, the New Zealand weevils demonstrate a local diapause response over a wide geographical area (35°39' S to 43°39' S), suggesting their introduction from a localized source in Argentina. Furthermore, the complete absence of any latitudinal effect on the critical photoperiod indicates no evolutionary change in diapause behaviour since their establishment. This lack of adaptive evolution, and perhaps the very existence of diapause itself, confirms that *H. bonariensis* is a recent addition to New Zealand's insect fauna, which has very few native diapausing species⁷.

The synchronous induction of reproductive diapause of this pest species has important practical implications. It means that any phenological management approach should be applicable throughout New Zealand without the need to establish and adjust for regional variation in biological responses.

Gametocytogenesis by malaria parasites in continuous culture

D. C. Kaushal*, R. Carter*, L. H. Miller* & G. Krishna†

*Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205

†Laboratory of Chemical Pharmacology, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205

Asexual proliferation of malaria parasites proceeds by multiplication of the parasites within red cells. Following rupture of the host cells the released merozoites re-invade other red cells. On re-invasion, a proportion of merozoites become, not asexual parasites but gametocytes, the sexual stages infective to the mosquito vectors. Conversion of asexual parasites to gametocytes occurs not only during natural infections but also in continuous *in vitro* culture as reported first by Trager and Jensen¹⁻⁴ and by others^{5,6}. We showed previously that the proportion of early intra-erythrocytic stages (ring stages) of *Plasmodium falciparum* which developed into gametocytes in culture was influenced by culture conditions⁷. Gametocyte formation was rare in conditions supporting rapid proliferation but frequent when parasite densities were static. We now show that nearly 100% of ring stages develop into gametocytes in response to 1 mM cyclic AMP in static cultures whereas in rapidly growing cultures few rings become gametocytes in response to cyclic AMP.

We have used the Z isolate of *P. falciparum* grown in continuous culture in human red cells as previously described¹ (see Table 1 legend). Complete development of *P. falciparum* gametocytes, either in culture or in man, takes approximately 10 days. During this time the gametocytes develop from ring forms into mature crescent-shaped parasites capable of undergoing gamete formation. One of the developmental stages of gametocyte maturation, the stage II gametocyte, is easily identified and occurs 48 h after ring formation. In our previous study⁷ we devised a method for the daily measurement of the rate of production of gametocytes based on the percentage of rings that develop into stage II gametocytes 48 h later. Using this method,

Received 23 April; accepted 13 May 1980.

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Table 1 Asexual parasitaemia and gametocyte production by *P. falciparum* in culture in the presence or absence of added cyclic AMP

Days after subculture		4	5	6	7	8	9
Rings*	No cyclic AMP	1.9±0.2	2.1±0.3	2.0±0.31	1.5±0.2	1.4±0.2	1.5±0.6
	With cyclic AMP	2.0±0.3	0.9±0.1	0.5±0.1	0.4±0.1	0.16±0.04	0.11±0.04
Stage II gametocytes*	No cyclic AMP	0.9±0.3	2.0±0.7	4.1±1.1	5.4±1.1	8.5±2.2	9.0±1.7
	With cyclic AMP	1.1±0.3	5.1±2.9	24.1±3.7	56.7±6.0	36.7±6.7	17.0±7.2
Stage III gametocytes*	No cyclic AMP	0.7±0.3	1.0±0.4	1.8±0.7	3.2±1.1	4.5±2.3	2.5±0.6
	With cyclic AMP	0.8±0.3	1.5±0.5	4.4±1.8	22.9±4.7	50.8±7.0	50.4±9.2
% Rings to gametocytes†	No cyclic AMP	2.5±0.7	3.3±0.6	4.4±0.6	6.1±1.1	—	—
	With cyclic AMP	11.4±2.9	58.1±8.2	70.8±5.7	37.7±18.4	—	—

Parasites were grown in human red cells in continuous culture using a method we have described previously¹ and based on the methods developed by Trager and Jensen² and by Haynes *et al.*³ Cultures were grown in tissue culture flasks (Corning, 25 cm², polystyrene, 25100) in type A, Rh+ human red cells at a 5% haematocrit in culture medium. The culture medium was made up in double distilled water from RPMI 1640 powdered medium with glutamine and without bicarbonate (Gibco) to which was added HEPES buffer (Sigma) to 25 mM. Immediately before use sodium bicarbonate was added to 24 mM and the medium was supplemented to 10% with inactivated human serum, blood type A, Rh+. The complete medium was filtered through a 0.45 µm Nalgene filter. No antibiotics were used. Culture flasks contained 10 ml of the parasitized blood cell suspension and were laid on their broad sides (25 cm²) to give a medium depth of 4 mm and a cell depth of 0.2 mm. Medium was replaced daily and the flasks gassed with a mixture of 3% CO₂, 6% O₂, 91% N₂, hermetically sealed and maintained at 37°C. Fresh cultures were established by diluting a growing culture with uninfected red cells at a 5% haematocrit in culture medium to give an initial parasitaemia of 0.1–0.5%. The diluted cultures were thereafter dispensed into flasks and maintained as described above. Four days after setting up a *P. falciparum* culture in fresh red cells and medium, each flask was divided and 3 ml of the culture suspension was placed into each of two new flasks. The flasks were placed on their smallest end to give a medium depth of 3.3 mm and a cell depth of 0.17 mm. One flask was cultured as before with normal medium; in the other the medium was supplemented with 1 mM cyclic AMP. Ring stages and gametocyte densities were estimated daily from blood smears stained with Giemsa's stain. Stage II and stage III gametocytes are defined by morphological criteria and are estimated to be 2–3 and 3–5 days old, respectively (see refs 4, 7, 10). Results are given as the mean ± 1 s.e. of 9 separate experiments.

*Parasite counts are expressed as the number of parasites (gametocytes or rings) per 100 red cells.

†The per cent of rings developing into stage II gametocytes 2 days later (see ref. 7 for full explanation).

we have studied the effect of cyclic AMP on the production of gametocytes in culture.

The mean densities of ring stages and gametocytes (stages II and III) during culture in the presence or absence of 1 mM cyclic AMP are shown in Table 1. Cyclic AMP was added and measurement of gametocyte production was begun after the parasites had been growing for 4 days in fresh red cells and medium. The percentage of rings which developed into gametocytes was 5–10 times greater in the presence of cyclic

AMP. The data show that 24 h after their appearance as stage II gametocytes, they progressed quantitatively into stage III gametocytes. Measurement of the high proportion of rings developing into gametocytes in the presence of cyclic AMP is not, therefore, an artefact due to the accumulation of stage II gametocytes in culture.

When cultures were exposed to various concentrations of cyclic AMP (Fig. 1), 1 mM cyclic AMP mediated the greatest increase in the proportion of rings forming gametocytes. Higher concentrations (above 1 mM, data not shown) were lethal to all stages of the parasite. At concentrations as low as 0.01 mM the proportion of rings forming gametocytes was still more than twice that of cultures without added cyclic AMP.

Dibutyl cyclic AMP, an analogue of cyclic AMP, was equally effective at equivalent concentrations in inducing gametocyte formation. Administration of 0.1 mM and 1 mM cyclic GMP and dibutyl cyclic GMP had no effect on gametocyte formation. To test the effects of degradation products of cyclic AMP, we administered 5'-AMP, 3'-AMP, ADP and ATP, adenine and adenosine at concentrations of 0.1 mM and 1 mM. None of these compounds influenced gametocyte formation.

The following experiment was carried out to test the effect of cyclic AMP on the proportion of rings which develop into gametocytes at different times during culture. A parasite culture was diluted with fresh red cells and medium to achieve a parasite density of 0.5% and the culture divided equally into seven flasks. To each flask 1 mM cyclic AMP was added starting on a different day. The proportion of rings which were developing into gametocytes was measured 24 h after the addition of cyclic AMP. During the first 3 days after subculture, when there was rapid growth of the asexual parasites (Fig. 2), the proportion of rings which formed gametocytes following addition of cyclic AMP remained low (<10%) and even fell slightly (Fig. 2). Prolonged exposure of the parasites to 1 mM cyclic AMP during this period of rapid asexual growth was uniformly lethal to the asexual parasites (data not shown). In cultures without added cyclic AMP the proportion of rings forming gametocytes typically fell to very low levels (Fig. 2). From the fourth day onward the rate of asexual proliferation was very low and degenerate asexual parasites began to appear in culture. During this time the proportion of rings which spontaneously developed into gametocytes began to increase again. Moreover, during this phase of the culture, addition of 1 mM cyclic AMP elevated the proportion of rings which developed into gametocytes to about 10 times the spontaneous rate (Fig. 2). By the sixth day in culture, when 10–15% of rings spontaneously developed into gametocytes, almost 100% of rings were stimulated to become gametocytes 24 h after the addition of 1 mM cyclic AMP.

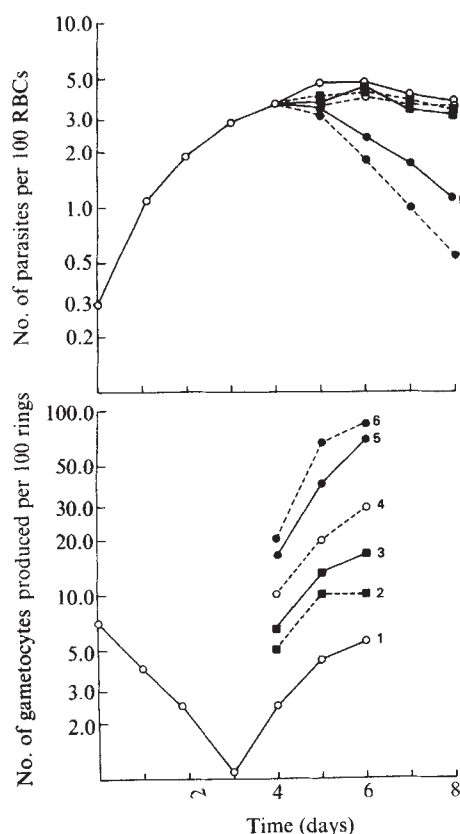


Fig. 1 The effects of different concentrations of cyclic AMP added from the fourth day of culture onward on the asexual parasitaemia and rate of gametocyte formation during *in vitro* culture of *Plasmodium falciparum* in human red blood cells RBCs. (1) No cyclic AMP added; (2) 0.01 mM cyclic AMP; (3) 0.05 mM cyclic AMP; (4) 0.1 mM cyclic AMP; (5) 0.5 mM cyclic AMP; (6) 1.0 mM cyclic AMP.

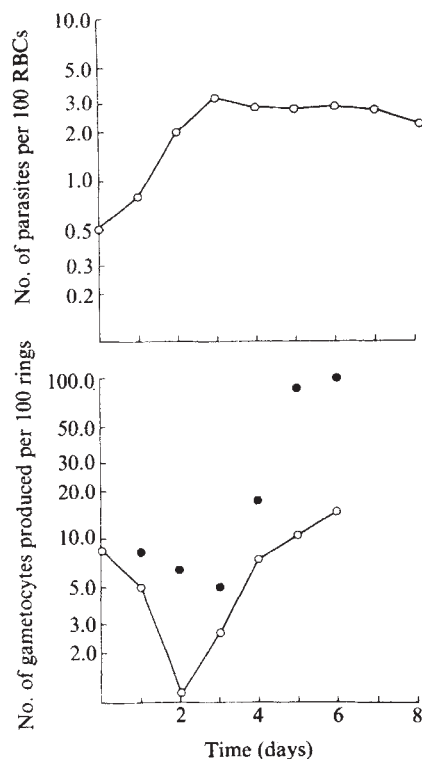


Fig. 2 The changing rate of formation of gametocytes from rings in response to cyclic AMP added on different days during the growth of *P. falciparum* in culture. A subculture of *P. falciparum*, set up by addition of fresh red cells and medium to a growing culture as described in Table 1 legend, was divided into 3-ml aliquots among seven flasks; the starting parasitaemia was 0.5%. One culture flask had no cyclic AMP added and showed the asexual growth and the daily rate of spontaneous formation of gametocytes from rings (○—○). To each of the other six flasks 1 mM cyclic AMP was added beginning on day 0 for flask 2, on day 1 for flask 3 to day 5 for flask 7. For each flask the rate of formation of gametocytes from rings was determined after the parasites had been exposed to cyclic AMP for 24 h (●). The rate of formation of gametocytes for flask 2 is shown on day 1, for flask 3 on day 2 and so on.

Our results demonstrate that the response to cyclic AMP by rings of *P. falciparum* in continuous culture changes according to the state of the culture. During rapid asexual proliferation, few rings develop into gametocytes in response to 1 mM cyclic AMP. Indeed, prolonged exposure to 1 mM cyclic AMP is lethal to the parasites. After rapid proliferation has ceased and the density of asexual parasites is constant, almost 100% of ring forms develop into gametocytes in response to 1 mM cyclic AMP. This change in responsiveness to cyclic AMP parallels the change in the rate of spontaneous gametocyte formation. Thus, during rapid asexual proliferation, spontaneous gametocyte formation falls to low levels or is absent; after asexual densities have become more or less constant, up to 10% of rings develop into gametocytes.

A similar change in responsiveness to a cyclic AMP stimulus was found in the amoeba *Dictyostelium discoideum*. In the presence of an adequate food source the free-living amoebae proliferated exponentially and did not respond to 0.001 mM cyclic AMP⁸. When the food supply became exhausted and exponential growth ceased, the amoebae became highly sensitive to cyclic AMP and formed aggregates in the early stages of spore formation. An analogous situation may apply to *P. falciparum* in culture. Parasites in cultures in which densities are not increasing are also in a state of near starvation; glucose levels become almost totally depleted before each addition of fresh culture medium. The state of starvation may, therefore, be associated with the development in the ring stages of a state of responsiveness to stimuli which induce gametocyte formation.

Received 28 November 1979; accepted 23 May 1980.

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Determination and cellular commitment in the embryonic amphibian mesoderm

D. Forman & J. M. W. Slack

Imperial Cancer Research Fund, Mill Hill Laboratories, Burtonhole Lane, London NW7 1AD, UK

In classical embryology, the term 'determination' was used to describe the process by which a region of the embryo became committed to form a particular part of the body and it was understood that there was a particular stage of development at which determination occurred with respect to each decision^{1–3}. Some authors^{4,5} insisted that only an irreversible commitment should be described as determination, but this leaves undefined the nature of the preceding events involved in its establishment. We show here that there may be several stages of commitment for an organ rudiment that can be distinguished from one another by the use of different methods for altering the pathway of development. We have investigated the region of the amphibian mesoderm that is destined to become somitic muscle in the course of normal development and applied to it a variety of isolation and grafting techniques. The results, which show an extensive period of lability within this region, can be used to explain how pattern formation might occur within the dorso-ventral axis of the mesoderm.

In the experiments described here, the prospective somite region of the axolotl (*Ambystoma mexicanum*) was treated in one of four different ways as shown in Fig. 1. (1) The region was dissected out, wrapped in an ectodermal jacket obtained from the anteroventral region of a stage 13 embryo, and cultured in a buffered salt solution for 10 days. (2) After dissection, the explant was dissociated into single cells and allowed to reaggregate into clumps for 48 h. The clumps were then wrapped in ectoderm and cultured as in treatment (1). (3) After dissection, the explant was grafted into another embryo of the same stage to replace a piece of ventral mesoderm and the host embryo was allowed to develop for 12 days. (4) The region was dissected out, dissociated and reassociated, and then grafted ventrally to a host embryo of the same stage as the donor had been at the time of explantation. The host was allowed to develop for 12 days.

The results of treatment (1) show that at both neurula and head process stages the isolated tissue behaves in a similar way to normal development in that it differentiates predominantly into muscle (Table 1a, Fig. 2a). According to this criterion, one could therefore describe the prospective somite region of the mesoderm as being determined by the neurula stage and, indeed, the result is consistent with classical work using both urodele and anuran embryos, which indicated that the determination of this region had occurred by the time of gastrulation^{6,7}. Some of the isolates also formed small amounts of pronephric tubules and/or mesenchyme. The formation of more than one tissue type is common in these experiments and we think that it may be the result of single cells or small groups of cells becoming detached from the main mass of tissue and behaving differently as a result. Because unwrapped controls remained undifferentiated, we believe that the ectodermal

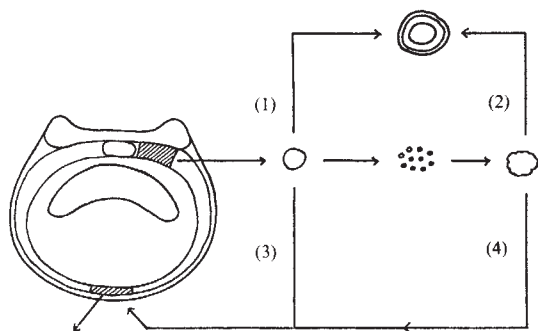


Fig. 1 The four experimental procedures. The diagram represents a transverse section through the trunk region of an axolotl neurula and shows the location of the prospective somite region (dorsal, shaded). This region was dissected out of the embryo on both sides with tungsten needles in $\frac{1}{4}$ strength 'normal amphibian medium' (NAM)¹¹ containing 0.05% trypsin. The explants were washed, fused and allowed to round up in full-strength NAM for 30 min. Operations were carried out on neurulae (stages 15–18)¹⁴, head process stages (21–24)¹⁴ and tail bud stages (26–28)¹⁴. By the head process stage the somite region had begun segmentation at the anterior end. (1) Explants were wrapped in ectoderm and cultured in NAM for 10 days. (2) Explants were dissociated by washing in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free NAM and then treated for 5 min with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free NAM containing 1 mM EDTA. The single cells were micropipetted into the centres of agar-coated 0.4-ml multiwell plates containing NAM. After 48 h of reaggregation, the resulting cell clumps were wrapped in ectoderm and cultured in NAM for 10 days. (3) A piece of tissue, about one-quarter of the size of the prospective somite file, was grafted into the ventral region of host embryos of the same stage. The embryos were allowed to develop in $\frac{1}{4}$ strength and later $\frac{1}{10}$ strength NAM for 12 days. (4) A piece of dissociated–reaggregated tissue was grafted ventrally as in (3). All cultures were kept at 18 °C and after the appropriate period, the isolates or host embryos were fixed and processed for histological examination. Details of the methods used for obtaining embryos, operational procedures and tissue identification have been given previously¹¹.

jackets are essential for the successful differentiation of these isolates.

Treatment (2) gave significantly different results (Table 1b). At both neurula and head process stage there was now a much reduced tendency for the isolates to differentiate into muscle. Instead, the main tissue found was pronephric tubules together with substantial amounts of mesenchyme (Fig. 2b). We feel that the most likely interpretation of this result is that undifferentiated cells which would have turned into muscle turn into pronephros instead. The difference cannot be due to the selective overgrowth of one committed cell population by another because the culture medium used in these experiments is not nutritive and although the cells undergo several cleavage divisions there is no net growth of the tissue. Such selection would therefore have to be accompanied by massive cell death, which we do not observe. Because some undifferentiated cells persist in the isolates after the culture period, the only possible explanation based on selection is that the differentiation of committed pre-kidney cells is inhibited in treatment (1) and the differentiation of committed pre-muscle cells is inhibited in treatment (2). We cannot exclude this explanation but we believe it to be unlikely. By the tailbud stage of development the lability of the tissue seems to be lost because muscle is

Table 2 Tissue types identified in grafted material following a graft of prospective somite region axolotl mesoderm to replace ventral mesoderm in a donor of equivalent stage

Stage at which graft performed	Cases	Muscle	Pronephros	Blood	Mesenchyme
<i>a</i> , Undissociated					
Neurula	16	4 (25%)	5 (31%)	15 (93%)	6
19/20	11	6 (55%)	1 (9%)	7 (63%)	3
Head process	10	9 (90%)	2 (20%)	7 (70%)	5
<i>b</i> , Dissociated–reaggregated					
Neurula	11	3 (27%)	7 (64%)	10 (91%)	4
Neurula control*	10	10 (100%)	1 (10%)	10 (100%)	2
Head process	10	2 (20%)	10 (100%)	4 (40%)	8
Head process control*	8	8 (100%)	3 (37%)	5 (62%)	1

* Controls were undissociated isolates cultured in salt solution for the time period during which dissociated cases were allowed to reaggregate.

then the dominant cell type formed by the re-aggregated isolates (Table 1b).

Prospective somite regions which were grafted to the ventral position while donor and host embryos were at the neurula stage (treatment (3)) differentiated predominantly into erythrocytes (Fig. 2c,d). Small amounts of muscle and pronephric tubules were also found in some of the grafts (Table 2a). At later stages of development the grafts gave rise to more muscle and fewer erythrocytes (stages 19/20 in Table 2a) and by the head process stage the grafts differentiated mainly into muscle (Fig. 2e) and the few erythrocytes which were formed were dispersed around the outside of the graft tissue. This type of switch from position-dependent to source-dependent development has been reported by Yamada⁸, although in his work on the newt *Cynops pyrrhogaster* the switch occurred rather earlier, at about stage 15.

If the prospective somite region is dissociated and re-aggregated before being grafted to a ventral position (treatment (4)), the period of lability is even longer (Table 2b). As with the re-aggregated isolates there was a much reduced differentiation into muscle and a much increased formation of pronephric tubules. There was also a significant formation of erythrocytes which was more extensive at the neurula than at the head process stage. Results with the undissociated controls confirm that these effects were not due to the fact that the re-aggregated isolates were cultured for 48 h before grafting. Indeed, the 48-h culture period for the controls actually reduced the lability of the neurula stage ventral grafts; cultured grafts always maintained their capacity for muscle differentiation whereas non-cultured grafts did not (compare Table 2a neurula with Table 2b neurula control).

For all the operations reported in Table 2a, b we are confident that the tissues we identify are genuinely derived from the graft and not from the host. This is because grafts to the ventral position persist as readily visible clumps of tissue for many days, as shown in Fig. 2c. Also, 21 of these grafts, including cases from each experimental class, were carried out using triploid donor embryos and these triploid cells were later identified in the graft (Fig. 2f). In axolotls the number of nucleoli per nucleus corresponds to the ploidy⁹ and so the identification of trinucleolar cells is proof of their donor origin. Nucleoli are readily visible in muscle and kidney cells and although they cannot be seen in mature erythrocytes they can be prominent in the erythrocyte precursor cells which are an intimate part of the graft tissue.

The results are summarized in Table 3.

It seems, therefore, that the prospective somite region is determined with respect to self-differentiation in isolation throughout the developmental period considered. However, the determination of the tissue can be modified by grafting to a ventral position up to the head process stage and can be modified by dissociation and reaggregation up to the early tailbud stage. This latter stage is the time at which muscle specific differentiation products can first be detected in the region (T. J. Mohun,

Table 1 Tissue types identified in isolated explants of prospective somite region of axolotl mesoderm

Stage from which tissue isolated	Cases	Muscle	Pronephros	Blood	Mesenchyme
<i>a</i> , Undissociated					
Neurula	8	7 (87%)	2 (25%)	0	6
Head process	13	13 (100%)	7 (54%)	0	9
<i>b</i> , Dissociated–reaggregated					
Neurula	15	6 (40%)	14 (93%)	0	15
Head process	12	2 (17%)	12 (100%)	0	10
Tailbud	8	7 (87%)	6 (75%)	0	7

R. Tilly, R. Mohun and J.M.W.S., unpublished data) and which we therefore consider to be the onset of cytodifferentiation. Thus, in this case there is no state of truly irreversible determination before cytodifferentiation.

Yamada¹⁰ suggested that the dorsoventral pattern of structures in the mesoderm was specified by a graded signal emanating from the dorsal end of the tissue, which is the prospective notochord. Mesoderm closest to the notochord would be programmed to form the somites, the next layer would be programmed to form the kidney and the most ventral layer to form the blood islands. Our own previous work¹¹ supports the idea of a dorsal signal for the specification of the somite region and the experiments reported here suggest that the maintenance of this determined state may require the preservation of intercellular contacts. The fact that erythrocytes can be formed by grafts to the ventral position but not in isolates suggests the existence of a second 'blood inducing' signal which is present in the ventral region of the neurula. This would imply that in the absence of all

Table 3 Summary of results

<i>a, Neurula</i>	<i>Isolate</i>	<i>Ventral graft</i>
No dissociation	Muscle	Erythrocytes
Dissoc./reagg.	Pronephros	Erythrocytes (pronephros)
<i>b, Head process</i>		
No dissociation	Muscle (pronephros)	Muscle (erythrocytes)
Dissoc./reagg.	Pronephros	Pronephros

The predominant tissue was found in >85% of cases and the minor tissue in >50% of cases.

signals the mesoderm would develop into kidney which is what we find in the isolated tissue after disaggregation and reaggregation.

Although this scheme of pattern formation is largely speculative, it is consistent with all our results and does bear some formal similarity to 'double gradient' models which have been advanced to explain other embryonic phenomena^{12,13}. Further experiments to test its validity are in progress.

Received 25 March; accepted 5 June 1980.

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A hypolipaeic factor from the corpus cardiacum of locusts

I. Orchard & B. G. Loughton

Department of Biology, York University, Downsview, Ontario M3J 1P3, Canada

The glandular cells in the glandular lobe of the corpus cardiacum (CC) of locusts are the source of adipokinetic hormone (AKH), a hyperlipaeic hormone which has been characterized, sequenced and synthesized¹⁻⁴. The release of AKH is under the synaptic control of axons within the nervi corporis cardiaca II (NCC II)⁵. We show here that a second factor with hypolipaeic activity is also under the control of axons within the NCCII. However, its release sites lie within the storage lobe. Insulin, at physiological doses equivalent to those found in vertebrates, mimics the action of the hypolipaeic factor, and axons which show immunoreactive staining with anti-insulin have been located within the storage lobe. The locust hypolipaeic factor and insulin seem to have some common molecular features.

The CC of locusts has proved an attractive resource for insect endocrinologists because of its anatomical division into storage (which is composed largely of the axon terminals of the median neurosecretory cells) and glandular (composed almost entirely of intrinsic neurosecretory cells) lobes. This has allowed the localization of the source of locust AKH¹⁻⁴ in the glandular lobe. The CC is supplied by two nerves, the paired nervi corporis cardiaca I (NCC I) which enter the anterior aspect of the storage lobe and expand to constitute the bulk of the storage lobe, and

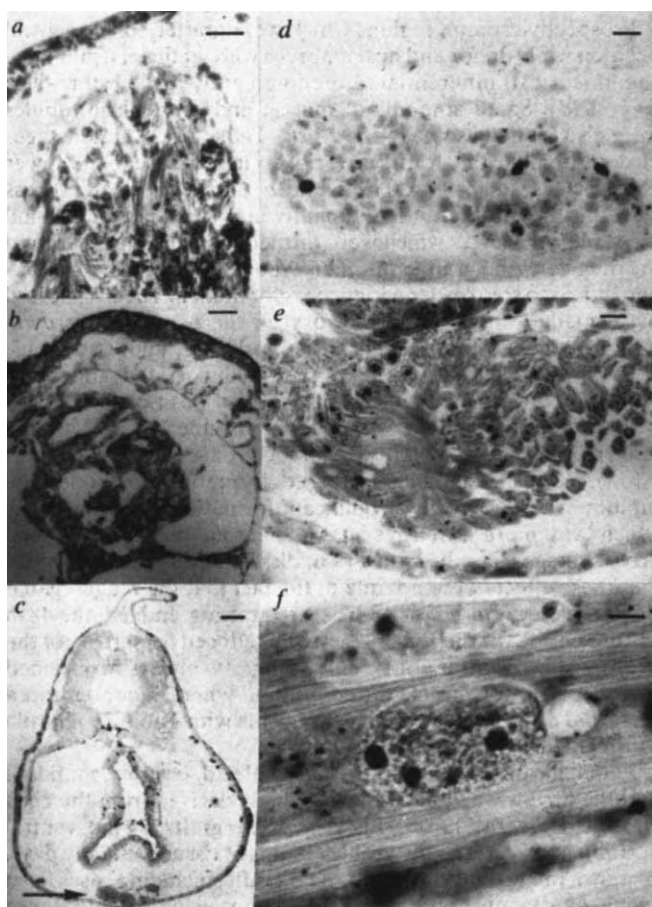


Fig. 2 *a*, Section through prospective somite region which was isolated from a neurula, wrapped in ectoderm and cultured for 10 days (treatment (1)). The tissue, although still containing yolk granules, has largely differentiated into muscle (scale bar, 50 μ m). *b*, Section through prospective somite region which was isolated from a head process stage embryo, dissociated and reaggregated, wrapped in ectoderm and cultured (treatment (2)). The tissue has formed pronephric tubules (scale bar, 50 μ m). *c*, Transverse section through the trunk region of an early larval stage axolotl which received a graft of prospective somite region into the ventral position (treatment (3)). The graft was carried out at the neurula stage, 12 days previously. The graft tissue is shown by an arrow (scale bar, 200 μ m). *d*, High power view of the graft indicated in *c*, showing erythrocytes (scale bar, 20 μ m). *e*, High power view of ventral graft of prospective somite region carried out at head process stage, showing muscle (scale bar, 20 μ m). *f*, Donor muscle cell in graft region autonomously marked by possession of three nucleoli in the nucleus. Tri-ploid donor embryos were obtained by a 10-min heat shock of 36 °C given 30-45 min after fertilization. The nucleoli are differentially stained by the Unna-Pappenheim stain (scale bar, 5 μ m).

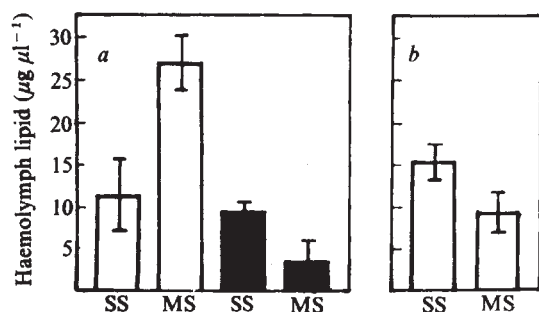


Fig. 1 *a*, Haemolymph lipid levels after injection of perfusate of the glandular lobe of the CC following its stimulation through the NCC II in the presence (solid bars) and absence (clear bars) of phenoxybenzamine. Note that the perfusate following maximal stimulation (MS, open bars) caused elevation of haemolymph lipid (hyperlipaemia) but that the perfusate following maximal stimulation in the presence of 10^{-6} M phenoxybenzamine caused a reduction in haemolymph lipid compared with controls (SS, open and closed bars). Release of hyperlipaemic hormone is obviously blocked in the presence of phenoxybenzamine but the release of hypolipaeic hormone is not. Mean $\pm$ s.e. of four assayed locusts. *b*, Haemolymph lipid levels after injection of perfusate of isolated storage lobe following stimulation of the NCC II. Note that the perfusate following maximal stimulation (MS) caused a reduction in haemolymph lipid (hypolipaeia). Mean $\pm$ s.e. of 12 assayed locusts. SS, Sub-threshold stimulation; MS, maximal stimulation.

the two nervi corporis cardiaca II (NCC II) which enter the storage lobe dorsally, close to the junction of storage and glandular lobes. We have recently demonstrated⁵ that stimulation of the NCC II causes the release of hyperlipaemic factors from the glandular lobe (see Fig. 1*a*, MS open bars).

During experiments on the pharmacology of the neurotransmitters mediating the release of hyperlipaemic factors, we observed preparations which, when stimulated through the NCC II in the presence of the α -adrenergic blocking agent phenoxybenzamine, caused a reduction in the haemolymph lipid of the insects studied (Fig. 1*a*, MS, closed bars). We interpreted this finding as indicating the continued release of a hypolipaeic factor, evident only when the release of the hyperlipaemic hormone(s)¹⁻⁴ was inhibited by blocking the synapse between NCC II axons and the intrinsic cells of the glandular lobe to the CC. To substantiate this hypothesis we examined the hypolipaeic activity in greater detail.

AKH activity has been associated primarily with the glandular lobe⁶; therefore, it was expected that the source of the hypolipaeic factor should lie elsewhere. Because incubation of the CC in phenoxybenzamine without nervous stimulation did not bring about release of hypolipaeic activity, it was assumed that its release was controlled by axons of the NCC II. Mason⁷ has shown that NCC II sends axons into both the storage and glandular lobes of the CC of *Schistocerca*. This was confirmed for *Locusta* by recording a propagated response from both storage and glandular lobes following stimulation of NCC II. Two approaches were adopted to confirm the presence of a hypolipaeic factor in the storage lobe of the CC. CCs were carefully dissected from mature adult female locusts so as to retain as much of the NCC I and NCC II as possible. Next, the glandular lobe was dissected away leaving the NCC II attached to the storage lobe. As several workers have pointed out⁸ that the boundary between storage and glandular regions is not discrete, we could not always be certain that all the glandular lobe had been removed. The storage lobe was placed in 100 μl of physiological saline⁹. The cut end of the NCC II was then drawn into a suction electrode and stimulated with impulses of sub-threshold current intensities at 5 Hz for 10 min. The bathing solution was collected and the preparation replaced with 100 μl of fresh saline. The procedure was repeated but the level of

stimulation was increased to bring about a maximally propagated response in the form of a compound action potential recorded from the internal face of the ventral lobe of the storage lobe of the CC. The control saline (collected after sub-threshold stimulation) and the experimental saline (following recording of compound action potentials) were assayed for hypolipaeic activity. Groups of four adult male locusts (10–14 days old) were each injected with 20 μl of the test saline. After 45 min the insects were bled through the cervical membrane. The lipid in the haemolymph was estimated gravimetrically⁵. In two out of three storage lobes stimulated in this way, the 'experimental saline' caused a 38% diminution of haemolymph lipid when compared with controls (Fig. 1*b*). Stimulation of NCC I failed to cause release of a hypolipaeic factor. We believe that in the third experiment the storage lobe might have retained some glandular lobe contaminants as lipid levels increased on injection of this preparation.

In a second series of experiments the ventral lobe of the storage lobe was dissected out of mature female locusts and homogenized in 100 μl of saline. Aliquots of the saline extract were injected into adult male locusts. Control insects received injections of saline or extracts of fat body. The lipid levels in the haemolymph of the test insects were determined 45 min later. Figure 2 shows the effects of injections of storage lobe extracts on the lipid levels in the haemolymph of test insects. It can be seen that as little as 0.0025 equivalents of the storage lobe

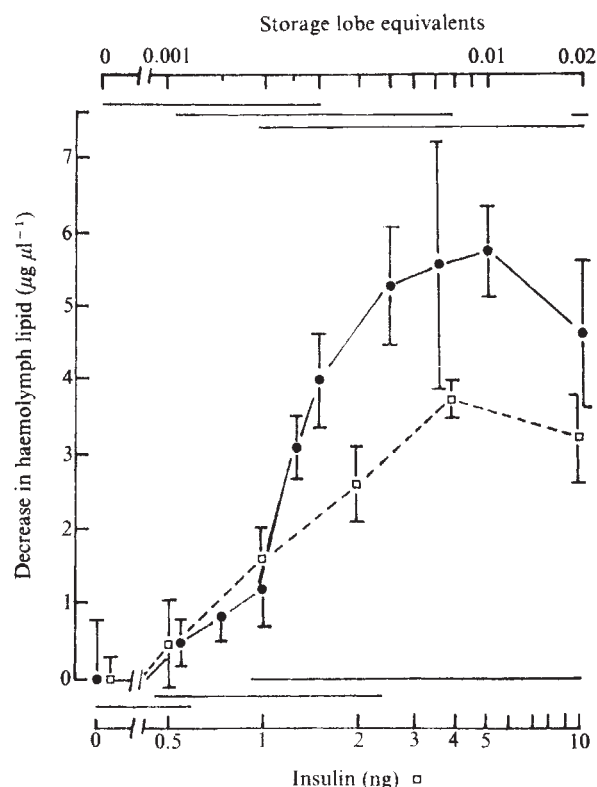


Fig. 2 Decrease in haemolymph lipid following injection of extracts of ventral lobe of the storage lobe of the CC or solutions of insulin (porcine). The resting level of lipid in the control insects for the storage lobe experiment was $10.1 \pm 0.8 \text{ g l}^{-1}$, giving a maximum hypolipaeic effect of 57% reduction in haemolymph lipid. Resting level in the insulin experiment was $6.6 \pm 0.3 \text{ g l}^{-1}$, giving a maximum hypolipaeic effect of 56% reduction in haemolymph lipid. The resting levels of lipid in the two sets of experiments reflect differences in the age and diet of the 'assay locusts'. The horizontal lines at the top and bottom of the figure denote homogeneous subsets formed by Student–Newman–Keul multiple range test ($\alpha = 0.05$) for storage lobe and insulin, respectively (results of one-way analyses of variance: for storage lobe, $F = 6.23$; d.f. = 9, 127; $P < 0.001$; for insulin: $F = 8.35$; d.f. = 5, 79; $P < 0.001$). The points are mean $\pm$ s.e.

caused a significant diminution of haemolymph lipid and that the hypolipaeic effect increased as higher concentrations of storage lobe were used.

In vertebrates, insulin decreases glucose levels in the blood and promotes lipid synthesis in the tissues. Several authors¹⁰⁻¹² have reported the presence of insulin-like molecules in insects. Furthermore, insulin has been shown to decrease the release of lipid from *Hyalophora* fat body *in vitro*¹³. Antibodies against porcine insulin (Sigma) were generated in rabbits and used to localize insulin immunoreactive substances in sections of brains and CC of locusts using the peroxidase-anti-peroxidase method¹⁴. Positive staining was detected in the ventral lobe region of the storage lobe and in median neurosecretory cells of the brain. The presence of insulin-like molecules in the CC prompted us to investigate the effect of insulin on the lipid levels in locust haemolymph. It can be seen from Fig. 2 that injection of insulin caused a marked decrease in haemolymph lipid. The similarity in the effect of extracts of storage lobe of the CC and insulin together with the presence of anti-insulin immunoreactive molecules in this part of the CC suggest the locust hypol-

ipaeic factor and insulin have some common molecular features.

Although the hypolipaeic factor has not been demonstrated in the haemolymph, the manner of its release from the CC suggests that the hypolipaeic factor in the storage lobe of the locust CC is indeed a newly discovered hormone. A slight hypolipaeia has been reported in cockroaches following injection of extracts of whole CC or corpora allata¹⁵ and Goldsworthy and Cheeseman¹⁶ suggested the possibility of such a hormone following the observation of slight hyperlipaemia in starved locusts in the absence of changes in AKH titre in the haemolymph. The physiological significance of the hypolipaeic factor could be to overcome the inhibition of glycolysis in flight muscle brought about by increased levels of diglyceride in the haemolymph¹⁶ or to restore haemolymph lipid levels to 'normal' following its dramatic increase during flight.

This work was supported by the National Research Council of Canada in the form of a Negotiated Development Grant to York University and NSERC grant A3645 to B. G. L. We thank Dr E. H. Miller for statistical analysis of the data.

Received 21 February; accepted 21 May 1980.

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Influence of voluntary intent on the human long-latency stretch reflex

J. C. Rothwell, M. M. Traub & C. D. Marsden

University Department of Neurology, Institute of Psychiatry and King's College Hospital Medical School, De Crespigny Park, Denmark Hill, London SE5 8AF, UK

In man, sudden stretch of an actively contracting muscle evokes a classical monosynaptic spinal reflex followed by an automatic 'long-latency' response, both evident as bursts of activity in the electromyogram (EMG)¹. This long-latency response, extensively investigated in the human long thumb flexor, but present in many other muscles², occurs too early to be voluntary and much indirect evidence suggests it represents the operation of a 'long-loop', perhaps transcortical, stretch reflex mechanism^{3,4}. Some investigators have found that the long-latency response may be modified substantially by the subject's intent, possibly by pre-setting of excitability levels within the central nervous system thereby influencing the 'long-loop' mechanism⁵⁻⁹. However, others have observed little effect of voluntary set on the automatic long-latency stretch response^{10,11}. It is notable that these studies, which have yielded conflicting results, have involved different muscles in a variety of experimental conditions. In some studies a warning signal preceded muscular stretch, while in others control trials were interspersed with stretches. To resolve the dilemma we have examined the influence of these variables, and found that the automatic long-latency response can be modified most strongly in conditions when the voluntary reaction time is shortest, for example, when the timing of the stimulus can be predicted accurately. We suggest that modification of a long-loop stretch response is not due to some central pre-setting process, but merely represents interaction between a reflex of long latency and a subsequent very rapid voluntary event, occurring early because of predictability of the stimulus.

We have investigated the effect of voluntary intent on the long-latency stretch response in flexor pollicis longus (FPL) and

biceps brachii of 10 normal volunteers. The experimental apparatus has been described previously^{1,2}. In the experiments on FPL, the thumb was clamped at the proximal phalanx and the subject maintained a constant position of the distal phalanx of the thumb against a background force (2.5 N) offered by a low inertia motor. For biceps, the subject rested his upper arm on a table top at chest height and maintained his semi-pronated forearm vertical against a constant force (8 N) exerted by means of a light chain attached to the motor and pulling on the wrist to extend the elbow. Surface EMGs were recorded by a PDP 12 computer after being rectified and integrated. In each subject muscle stretch was produced every 10-20 s by sudden increases in motor force lasting 1 s (to 5 N for FPL; 24 N for biceps). These disturbances were administered in three separate ways (*a*, *b* and *c* given in random order); (*a*) 500-650 ms after a warning bleep, the time being set by a pseudo-random generator; (*b*) a constant 8 s after the bleep; and (*c*) as in condition *a* but with 50% of randomly selected controls (unrecorded) in which the bleep was not followed by a change in motor torque. Before each warning signal the subject was instructed in random sequence either to 'let go' by relaxing completely, or to 'resist' by flexing as hard as possible on perceiving the disturbance. It was stressed that he was to react to the stretch and not to the earlier bleep signal. The computer recorded each trial over a period of 250 ms before the disturbance. Sixteen 'let go' and sixteen 'resist' trials were averaged separately in each group. The duration of the long-latency response was measured from the rectified EMG records of the 'let go' trials in condition *c*. The size of the response was measured from the integrated EMG records as the percentage increase in activity over control levels extrapolated from the first 50 ms of the sweep.

It has been shown previously that when a stimulus is preceded by a warning signal, the simple reaction time increases with the length and variability of the fore-period¹². Thus we expected the reaction time to be shortest in condition *a* in which the subject could predict the timing of the stretch stimulus most accurately. It was in this condition that the subject could voluntarily influence the size of the long-latency stretch response most effectively (Figs 1, 2). The addition of control trials (condition *c*)

or increasing the length of the fore-period to 8 s (condition *b*), both of which made the exact timing of the disturbance less certain, reduced the influence of voluntary intent on the stretch response by a similar amount.

Interestingly, there was a considerably greater effect of voluntary set on the stretch response in biceps than in FPL. This was unlikely to be due to a difference in voluntary reaction time because the latency of the EMG response to an auditory stimulus was longer in biceps than in FPL (biceps $151 \text{ ms} \pm 7.3 \text{ s.e.m.}$ ($n=8$); FPL 133 ± 7.7 ($n=8$); paired Student's *t*-test, $P < 0.005$). It is possible that because of the low inertia of the distal phalanx of the thumb, the stretch stimulus caused a relatively large and rapid change in length in FPL thus saturating the reflex

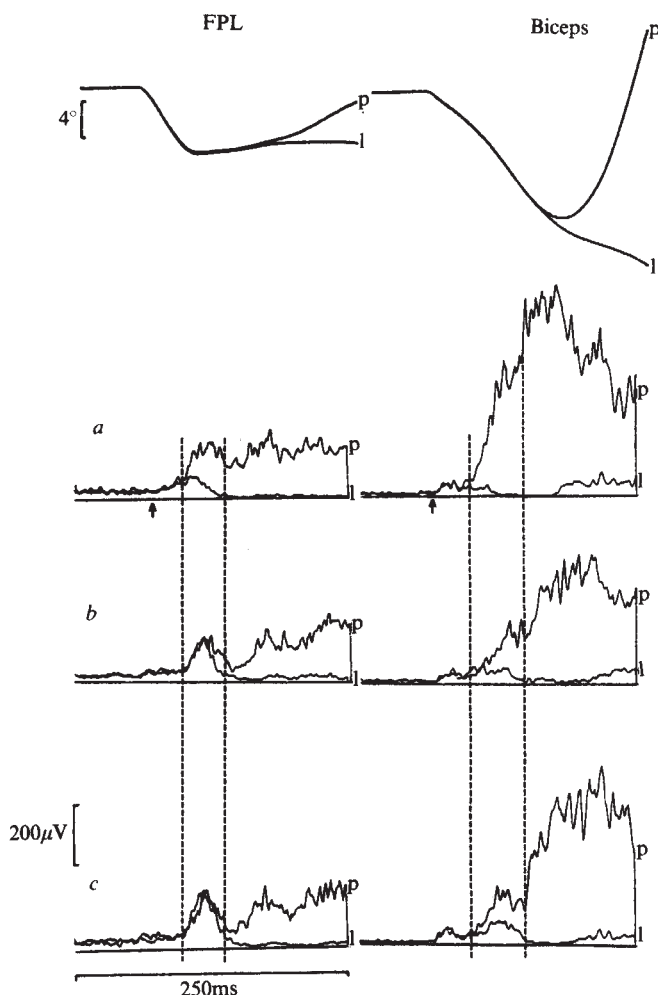


Fig. 1 The effect of prior instruction on the automatic long-latency response to muscle stretch in flexor pollicis longus (left-hand traces) and in biceps (right-hand traces) in a single subject. The top records illustrate the angular position of interphalangeal joint of the thumb and the elbow, the initial positions of the joints being 10° and 90° flexion respectively. The lower three traces show rectified EMG. The motor torque was increased 50 ms after the start of each sweep and the subject was instructed before each trial to 'resist' (p) or 'let go' (l) on perceiving the disturbance. Each trace shows the average of 16 p trials superimposed on the average of 16 l trials. The three experimental designs, *a*, *b* and *c*, performed as described in the text, in the sequence *b*, *c*, *a*. Position traces (top) are from trial *a*. The duration of the long-latency stretch response (vertical dotted lines) was estimated from the 'let go' (l) trials in trace *c*. A vertical arrow marks the onset of a small spinal latency component of the response with a latency of about 25 ms in FPL and about 15 ms in biceps.

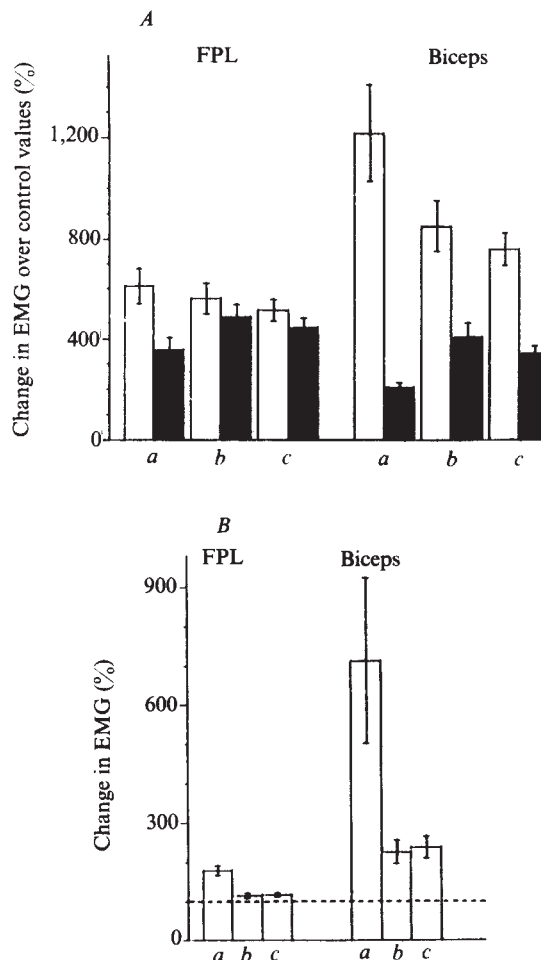


Fig. 2 *A*, Average size of the long latency stretch reflex (± 1 s.e.m.) in FPL and in biceps of 10 subjects in the three different experimental conditions *a*, *b* and *c* (see text). Open bars indicate the size in the 'resist' (p) trials; hatched bars the 'let go' (l) trials. Size of reflex EMG was measured from integrated records and is expressed as a percentage of basal levels of activity calculated for the same duration as the reflex, but extrapolated from the control period immediately before the stretch stimulus. *B*, Mean percentage change (± 1 s.e.m.) in the size of the stretch reflex during the 'resist' trials compared with the size in the 'let go' trials for each experimental condition (see text). The horizontal dotted line at 100% indicates that the reflex size was the same in both conditions.

output (see Fig. 1). However, when a smaller thumb disturbance was given (increase in force from 2.5 to 3.0 N) in condition *a*, the influence of voluntary set was reduced, presumably because of the increase in voluntary reaction time to the smaller stimulus.

In a subsidiary experiment the smaller stretch was administered together with a simultaneous electric shock (60 V; 50 μs) to the contralateral median nerve at the wrist (voluntary reaction to the electrical stimulus alone was $100 \text{ ms} \pm 2.8 \text{ s.e.m.}$) so as to shorten the reaction time to a small non-saturating stretch stimulus. Now the influence of voluntary intent was enhanced to levels greater than observed in the conditions described previously for larger stretches (Figs 2, 3). In contrast, a simultaneous electric shock had no apparent effect on reflex size when given with a larger stretch when reaction time was already short (Fig. 3).

In conclusion, the degree of voluntary modification of the size of the long-latency automatic stretch response depends on factors which affect reaction time. The relatively small effects of

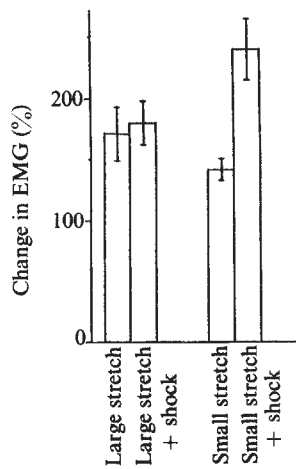


Fig. 3 Average percentage change (± 1 s.e.m.) in the size of the long-latency stretch reflex in FPL of ten subjects during the 'resist' and 'let go' conditions when the stretch stimulus was given alone or paired with a simultaneous electric shock to the contralateral median nerve at the wrist. All trials performed under condition a, with a warning signal given 500–650 ms prior to the muscle stretch. Left bars, large stretch (from 2.5 to 5 N); right bars, small stretch (from 2.5 to 3 N). The electric shock was arranged to be above threshold for thenar muscle contraction.

voluntary intervention seen in FPL may be because it is impossible to administer a stretch stimulus which is both easily and therefore rapidly perceived, and yet which does not saturate the stretch reflex mechanism. In contrast, the stretch response in biceps does not saturate so easily (because the inertia of the arm precludes very rapid stretches with the apparatus used), so that the influence of rapid voluntary intervention is seen more easily than in FPL. Prior instruction, rather than pre-setting excitability levels in the long-loop pathways, seems to influence the automatic long-latency response to stretch by superimposing fast descending voluntary activity on the earlier automatic event.

Received 11 March; accepted 6 June 1980.

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Interaction of anaesthetics with electrical synapses

M. F. Johnston, S. A. Simon & F. Ramón*

Departments of Physiology and Anaesthesiology, Duke University Medical Center, Durham, North Carolina 27710

Studies of the interaction of anaesthetics with various preparations, from whole animals to organic solvents, have been continuing since Overton and Meyer found a correlation between anaesthetic potency and solubility in olive oil¹. Although the physiological basis of anaesthesia is far from clear, one popular hypothesis is that anaesthetics act primarily by interfering with the normal functioning of chemical synapses^{2–4}. This hypothesis is supported by experiments showing that these synapses are more sensitive to both local and general anaesthetics than are axons. The effects of anaesthetics on electrical synapses (gap-junctions or nexus) have not previously been studied. These ubiquitous structures, presumably responsible for cell-to-cell communication⁵, are found in most vertebrate and invertebrate tissues. We report here the effects of several anaesthetics on electronic coupling between nerve cells, and show that electrical synapses are less sensitive to most anaesthetics than are chemical synapses and axonal membranes.

Experiments were performed on segmented giant axons of the nerve cord of crayfish (*Procambarus clarkii*). Each axonal segment is coupled to its neighbours by bidirectional electrical synapses near each segmental ganglion^{6,7}. Measurements were made at the second or third abdominal ganglion. Intercellular coupling was determined by injecting hyperpolarizing square pulses of current and measuring the voltage responses at both sides of the intersegmental septum with intracellular microelectrodes. Septal resistance (R_s) and axon membrane resistance (R_m) were calculated from these measurements^{7–9}. Propagated action potentials (p.a.ps) were also recorded as they travelled through the septal region. Control values for resting membrane potential and action potential amplitude are 88.9 ± 2.0 mV and

123.8 ± 3.8 mV (mean $\pm$ s.d.), respectively. Septal resistance and membrane resistance averaged 40 ± 17 K Ω and 188 ± 18 K Ω , respectively.

Axons were bathed in a modified van Harreveld solution containing 205 mM NaCl, 13.5 mM CaCl₂, 5.4 mM KCl, 2.6 mM MgCl₂, and 5 mM HEPES, adjusted to pH 7.5. Anaesthetics, with the exception of ketamine which was obtained from Parke-Davis, were purchased from Sigma. All drugs were added extracellularly unless otherwise stated. The standard internal solution (SIS) contained 15 mM NaCl, 109 mM KF, 37 mM K citrate, 96 mM mannitol and 1 mM HEPES, adjusted to pH 7.5. The more water-soluble anaesthetics were added directly to the external solution. Octanol and nonanol were added to the external solution from 1M stock solutions in dimethylsulphoxide (DMSO). The final concentration of DMSO was always less than 0.5% (v/v), which by itself had no effect on electrical behaviour. Drug concentrations were increased until the axon would no longer maintain a propagated action potential. Experiments were performed at 15°C.

Figure 1 summarizes data obtained when heptanol (3 mM) was applied externally. This figure shows propagated action potentials and the voltage-current relationships of the septal region during control (a, d), heptanol (b, e) and recovery (c, f). Data for other anaesthetics at various concentrations were obtained similarly.

Figure 1b shows that 3 mM heptanol blocks propagation of action potentials across the septum. These effects of heptanol are reversible (Fig. 1c). Blockade of action potentials can be explained, from septal V-I relationships, by an increase in resistance from 50 K Ω (Fig. 1d) to 600 K Ω (Fig. 1e). In these data only the V-I curves in the third quadrant (hyperpolarization) are relevant. The nonlinearity of the V-I relationships seen in the first quadrant is due to changes in membrane resistance during axon depolarization. Under voltage-clamp conditions the V-I relationships are linear over a voltage range of ± 100 mV^{8,9}. In Fig. 1g, the septal resistance obtained from the slope of the V-I relationships is plotted against time. Arrows at 30 and 80 minutes indicate the beginning and end of heptanol perfusion, respectively.

Table 1 summarizes the results obtained with local and general anaesthetics with regard to their effect on p.a.ps (column

* To whom correspondence should be addressed.

Table 1 Effect of anaesthetics at the crayfish septal synapse

Anaesthetic	Concentration (mM)	Effect on p.a.ps	Coupling at synapse	Reversibility
<i>Local</i>				
Procaine	5.0	blocked	normal	++++
Benzocaine	3.0	blocked	normal	++++
Propranolol	1.0	blocked	normal	++
<i>General</i>				
Ketamine	2.0	delayed	normal	++++
<i>n</i> -Hexan-1-ol	10.0	blocked	slightly decreased	++++
<i>n</i> -Heptan-1-ol	3.0	blocked	uncoupled	++++
<i>n</i> -Octan-1-ol	1.0	blocked	uncoupled	+++
<i>n</i> -Nonan-1-ol	0.3	blocked	slightly decreased	+++

Scale: + + + +, completely reversible; +, irreversible.

3), ability to electrically uncouple the axons (column 4), and reversibility (column 5). Table 1 shows that ketamine, procaine, propranolol (all of which exist in both the charged and neutral form at pH 7.5), and benzocaine (which is uncharged at pH 7.5), produced no significant changes in septal resistance despite their profound effects on p.a.ps. For the homologous series of *n*-alcohols, from hexanol to nonanol (C6–C9), the bulk concentrations correspond to approximately equal membrane concentrations¹. Alcohols from C6 to C9, all of which block action potentials at the concentrations used, affect septal resistance with a marked dependence on alcohol chain length. Specifically, heptanol and octanol dramatically increased septal resistance whereas hexanol and nonanol produced only minimal changes (Fig. 2). For example, octanol applied externally increased junctional resistance from 40 K Ω to 1.3 M Ω . All the anaesthetics reduced the resting membrane potential by 2–10 mV.

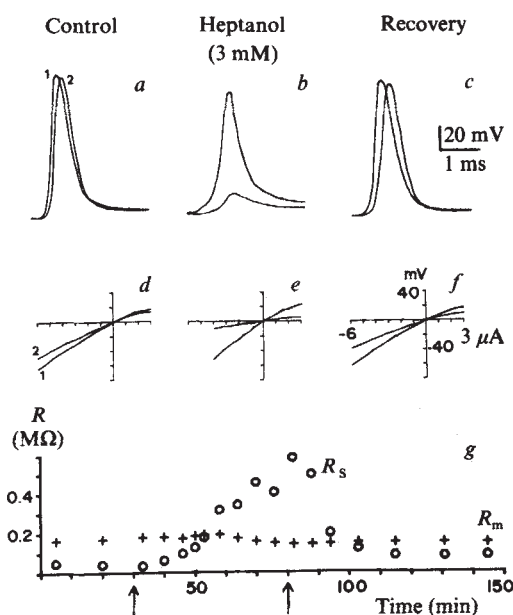


Fig. 1 Propagated action potentials (a–c) and voltage–current relationships (d–f) in the septal regions of an axon externally perfused with heptanol (3 mM). Data were obtained using three microelectrodes, one for current injection and the others for voltage measurements across the septum. Calibrations at c apply also to a and b, and those on f also to d and e. The small numbers beside the action potentials in a and the V–I relationships in d indicate (1) pre- and (2) postsynaptic recordings respectively. g shows septal (R_s , circles) and membrane (R_m , crosses) resistances calculated from the V–I relationships in the hyperpolarizing direction. Arrows at 30 and 80 min indicate the beginning and end of heptanol perfusion.

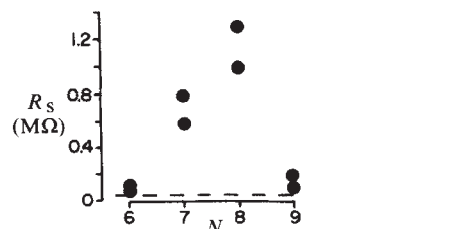


Fig. 2 Maximum septal resistance reached during external perfusion with hexanol (10 mM), heptanol (3 mM), octanol (1 mM) and nonanol (0.3 mM). Abscissa is alkyl chain length (N) of the alcohol. Results from two separate experiments are shown for each alcohol. The horizontal dashed line depicts the control value for septal resistance.

As octanol produced the greatest increase in R_s , additional experiments were performed to determine its 'site' of action. These experiments were performed using a technique previously developed to perfuse the interior of segmented axons^{8,9}. When one of the axons was perfused with SIS and 1 mM octanol was added to the external solution, uncoupling was similar to that described above. When 10 mM octanol was perfused internally the action potential was blocked as expected¹⁰; however, the septal resistance remained unchanged.

The above results have implications for mechanisms of anaesthesia. In addition they provide information regarding modulation at electrical synapses: (1) from Table 1 it is clear that electrical synapses are less sensitive to representative anaesthetics than either chemical synapses or axonal membranes, virtually eliminating them from the scope of structures that might be involved in anaesthesia. (2) The lack of effect of certain anaesthetics on electrical synapses and the chain length effect exhibited by alcohols serve to exclude several possibilities regarding mechanisms responsible for uncoupling. Positively charged molecules propranolol, ketamine and procaine (at pH 7.5) and the neutral molecule benzocaine had no effect on septal resistance. Thus, generalized membrane changes due to surface charges^{11,12}, dipole potentials^{13,14}, thickness¹⁴, tension²⁸ and membrane fluidity^{15,16} produced by these molecules apparently do not affect the operation of electrical synapses. For many preparations the homologous series of alcohols behave in a manner consistent with their solubility in membranes. This is the case for axons^{10,17}, phospholipid bilayers^{18–21}, monolayers²², red blood cells¹ and synapses in the abdominal ganglia of *Aplysia*²³. Such is not the case for the electrical synapse we investigated. A different alcohol chain length behaviour was described by Gage *et al.*^{25,26} on chemical synapses. These authors measured the time constants of decay of miniature end plate currents and found that the normal alcohols from C1 to C6 increased the time constant to decay, whereas octanol decreased it. Similarly, Richards *et al.*¹⁸ showed that no simple correlation exists between an alcohol's ability to block nerve conduction and to perturb bilayer structure.

The striking differences in potency of these alcohols in uncoupling the crayfish synapse implies an interaction which is strongly dependent on the alkyl chain length. Furthermore, the persistence of coupling when octanol is applied internally suggests that this 'site' is accessible only from the external medium. This chain length effect does not exclude a possible role for the hydroxyl group (or other polar groups) in the uncoupling mechanism. It would be of general interest to know, in light of current models of electrical synapses²⁷, how heptanol and octanol act to uncouple these synapses.

We thank Professors P. B. Bennett, M. Harmel and J. W. Moore for their support. This project was supported by grants HL-22767 and HL-12157.

Received 14 April; accepted 28 May 1980.

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Diffusion of acetylcholine in the synaptic cleft of normal and myasthenia gravis human endplates

S. G. Cull-Candy, R. Miledi & O. D. Uchitel

Department of Biophysics, University College London,
Gower Street, London WC1E 6BT, UK

When acetylcholinesterase (AChE) is inactivated the time constant of decay of the miniature endplate current ($\tau_{m.e.p.c.}$) depends on the lifetime of the endplate channel and on the speed of clearance of acetylcholine (ACh) from the synaptic cleft^{1–3}. The latter will be influenced by the binding of ACh to receptors, hence by their density and by the proportion of the ACh packet which attaches to them. By comparing $\tau_{m.e.p.c.}$ at various levels of receptor density, for example, following curare treatment, it is possible to estimate the proportion, p , of the ACh packet which normally attaches to the receptors¹. We have estimated p at the normal human endplate and compared this with myasthenia gravis (MG) affected human endplates where, as a result of reduced receptor density, there is a reduction in miniature endplate potential (m.e.p.p.)⁴ and m.e.p.c. amplitudes^{5,6}, α -bungarotoxin (α -Btx) binding^{7,8} and ACh-sensitivity⁹. The properties of the ACh-induced channels are similar at normal and MG endplates^{5,10} and it is unlikely that normal or MG channel properties are markedly altered by the various drug treatments used here^{11,12}. Therefore, at treated normal and at MG endplates any reduction in the time constant of decay of m.e.p.c.s indicates that the speed of clearance of the ACh from the cleft is increased. In the experiments reported here the proportion of the ACh packet which binds is estimated to be about 0.5 at normal human endplates. At MG endplates in the absence of cholinesterase activity m.e.p.c.s decay more rapidly than normal and the proportion of the ACh packet attaching is reduced.

Human intercostal nerve muscle was obtained from myasthenia gravis and control patients. The preparation of muscle bundles and electrophysiological techniques for intracellular recording and voltage clamping were as previously described^{5,6}.

Application of neostigmine to human endplates increases the amplitude of the spontaneously occurring m.e.p.p.s by inhibiting cholinesterase activity. The amplitude of m.e.p.p.s and the time constant of decay of the miniature currents have been studied over a range of neostigmine concentrations to determine the optimum level required to inhibit cholinesterase. This was found to be 10^{-7} to 5×10^{-7} g ml⁻¹ neostigmine; higher concentrations reduce the m.e.p.p. and m.e.p.c. amplitude. The amplitude and

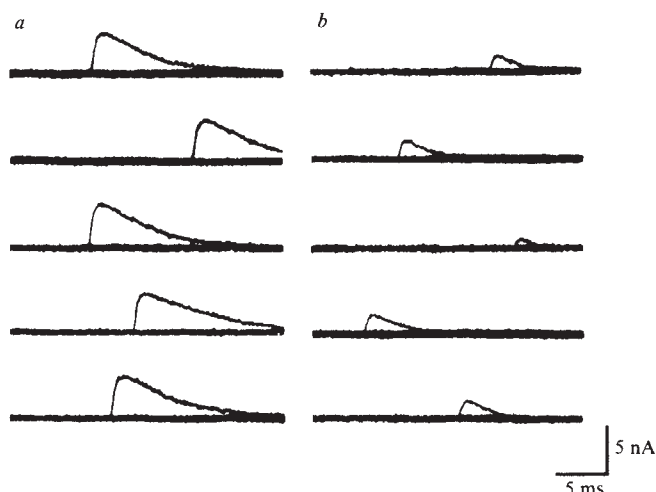


Fig. 1 M.e.p.c.s recorded from a single normal human endplate in normal medium in the presence of neostigmine (5×10^{-7} g ml⁻¹) (a) and after application of tubocurarine and neostigmine (5×10^{-7} g ml⁻¹) (b). In the presence of TC the m.e.p.c. amplitude is reduced and the time constant of decay of the m.e.p.c.s becomes briefer. M.e.p.c.s are recorded under voltage clamp ($V_m = -80$ mV, $T = 22^\circ\text{C}$) through a 500-Hz low pass filter which prolongs the rising phase and slightly attenuates the amplitude. The rising phase of m.e.p.c.s is also prolonged by the presence of neostigmine. Tubocurarine is applied iontophoretically. Tetrodotoxin (5×10^{-7} g ml⁻¹) was present throughout.

time course of m.e.p.c.s were obtained at normal human endplates equilibrated in neostigmine (5×10^{-7} g ml⁻¹). Tubocurarine (TC) was then applied either iontophoretically or in the bathing medium (10^{-6} g ml⁻¹). At this concentration TC has no appreciable effect on endplate channel properties in frog muscle¹¹. In addition, in some experiments α -Btx (5×10^{-7} g ml⁻¹) was used to block receptors partially, because even at doses higher than those used here it does not appreciably alter the characteristics of the remaining endplate channels¹. M.e.p.c.s were sampled during progressive block or during the recovery stage.

Figure 1 shows examples of normal m.e.p.c.s in neostigmine and m.e.p.c.s at the same endplate following iontophoretic application of TC ($V_m = -80$ mV). The reduction in m.e.p.c. amplitude, to less than one-third the normal size in this example, is accompanied by a decrease in $\tau_{m.e.p.c.}$ as previously reported for partially blocked endplates in other species^{1,13,14}.

The ratio of control to curarized $\tau_{m.e.p.c.}$, which in anti-esterase-treated endplates reflects the relative rates of clearance of ACh from the synaptic cleft, was obtained either from a semilogarithmic plot of the m.e.p.c. decay phase or from power density spectra of m.e.p.c.s. Figure 2 illustrates power spectra of m.e.p.c.s obtained from a single voltage-clamped human endplate in normal medium (neostigmine 5×10^{-7} g ml⁻¹) and after bath application of TC. $\tau_{m.e.p.c.}$ is obtained from the half-power frequency, f_c , at which the spectral density is reduced to one-half of the zero-frequency asymptote, according to $\tau_{m.e.p.c.} = 1/(2\pi f_c)$. In the examples in Fig. 2 the frequency shift from 27 Hz in normal medium to 35 Hz in TC indicates a decrease in $\tau_{m.e.p.c.}$ from 5.89 ms to 4.54 ms on partial curarization.

It has been suggested¹ that if a fraction, p , of the ACh molecules in the cleft binds to receptors, diffusion will be slowed down by the factor $1/(1-p)$. Then from the ratio of the time constants of the control and curarized m.e.p.c.s, τ/τ_{TC} , and the ratio of their amplitudes, α , it is possible to obtain an estimate of p : $p = (\tau - \tau_{TC})/(\tau - \alpha\tau_{TC})$.

$$p = (\tau - \tau_{TC})/(\tau - \alpha\tau_{TC})$$

Several kinetic assumptions involved in this relationship have previously been considered^{1,14}. The fraction of the packet which attaches is reduced to αp by reducing the number of available receptors, for example, during curarization or at the human MG endplate, where the density of receptors is reduced by an anti-receptor antibody (reviewed in refs 15, 16). Thus, when p is known at untreated endplates it should vary as αp for different levels of receptor block. In the experiment in Fig. 2 the fraction, p , of the ACh packet attaching to receptors is 0.41. The range of values for p , obtained by treating with TC or α -Btx, was 0.4–0.6 at normal human endplates; the mean value for p was 0.56 ± 0.05 ($\pm$ s.e., five endplates from three muscles).

We have found no significant difference between $\tau_{m.e.p.c.}$ at normal and MG endplates in normal bathing medium (that is, in the absence of esterase inhibitors) examined in four normal and four MG muscles (Table 1, see also ref. 10). On addition of neostigmine the decay time of both normal and MG m.e.p.cs increased markedly. For normal endplates $\tau_{m.e.p.c.}$ increased about three times whereas for MG endplates it was approximately doubled. Therefore, in the presence of neostigmine MG m.e.p.cs decay more rapidly than normal m.e.p.cs: $\tau_{m.e.p.c.}$ (normal) = 5.56 ms and $\tau_{m.e.p.c.}$ (MG) = 3.65 ms ($V_m = -80$ mV, $T = 22^\circ\text{C}$). These values are significantly different, $P < 0.001$ (see Table 1). The simplest explanation for such a difference between normal and MG m.e.p.cs is that the rate of diffusion from the cleft is faster at MG endplates as a result of the reduced ACh-receptor density.

If we compare the mean value of $\tau_{m.e.p.c.}$ for myasthenia gravis and normal m.e.p.cs in neostigmine (Table 1), where the ratio of the mean m.e.p.c. amplitudes, $\alpha = 0.35$ (range of values for α at 20 endplates, 0.17–0.46), the fraction of the packet attaching at the normal endplate is estimated to be $p = 0.45$ (range of values at 20 endplates, 0.38–0.60). This is assuming that the reduced amplitude and rapid time course of MG m.e.p.cs results mainly from a decreased receptor density at MG endplates. Furthermore, the fraction of the packet, αp , expected to attach at MG endplates is about 0.16 (range of values, 0.08–0.20). We have recorded MG m.e.p.cs before and after treatment with TC or α -Btx. In these conditions m.e.p.cs become so small that accurate estimates of $\tau_{m.e.p.c.}$ are difficult to obtain. However, it was possible to obtain values at five MG endplates at a clamp potential of -120 mV. This increased the amplitude of the m.e.p.cs and because of the voltage sensitivity of the channel lifetime¹⁰ caused a slight prolongation of $\tau_{m.e.p.c.}$. Although there has been no detailed study on the dependence of p on membrane potential, from the information available no marked change is

expected. Table 1 shows typical values from a myasthenic endplate treated with α -Btx in which $p = 0.17$. The range of values of p obtained was 0.1–0.2; the mean value for p was 0.16 ± 0.03 ($\pm$ s.e., five endplates in two muscles).

Therefore, the similarity in estimates of p (in the absence of esterase activity) obtained by curarizing normal endplates or by comparing normal with MG miniature currents, indicates that the curarized normal endplate resembles the MG endplate in certain respects. The fact that MG miniature currents decay faster than normal miniature currents may be explained by a loss of 'ACh binding sites' which results in a more rapid escape of ACh from the synaptic cleft. This renders unattractive the possibility that at MG endplates there is a decrease in the number of functional channels without a concomitant decrease in ACh binding sites. The reduced fraction of the ACh packet attaching at MG endplates falls within the range expected from the reduction in receptor density estimated from measurements of m.e.p.c. amplitudes. This suggests that the affinity of the receptors for ACh is similar at normal and MG endplates. It therefore seems that the presence of anti-receptor antibody on the postsynaptic membrane^{17,18} or changes in the endplate geometry associated with myasthenia^{9,19} do not have a predominant role in determining the time course of ACh diffusion out of the synaptic cleft.

We conclude that the fraction of the ACh packet attaching at anti-esterase-treated human endplates is similar to that previously reported for frog^{1,20} and rat¹² endplates. In addition, the fraction of the ACh packet attaching at MG endplates seems to be about one-third of that at the normal human endplate, due to a reduction in available ACh receptors. This results in a faster than normal clearance of ACh at the MG endplate.

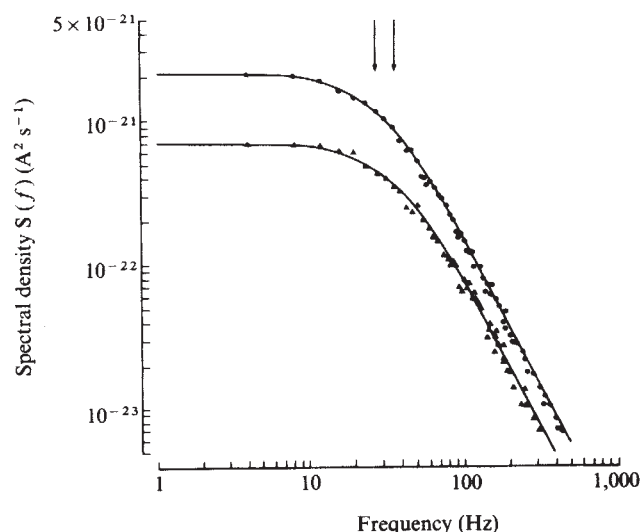


Fig. 2 Effect of tubocurarine on power density spectra of normal human m.e.p.cs from a single voltage-clamped endplate. ●, m.e.p.cs in normal medium, in the presence of neostigmine (2×10^{-7} g ml⁻¹) at a clamp potential $V_m = -80$ mV, $T = 22^\circ\text{C}$. Averaged spectrum of 12 individual m.e.p.c. spectra. Cut-off frequency indicated by arrow is $f_c = 27$ Hz, hence $\tau_{m.e.p.c.} = 5.89$ ms. ▲, m.e.p.cs treated with tubocurarine (10^{-8} g ml⁻¹) applied in the bathing medium) in the presence of neostigmine (2×10^{-7} g ml⁻¹), $V_m = -80$ mV, $T = 22^\circ\text{C}$. Averaged spectrum of 13 m.e.p.c. spectra. Cut-off frequency indicated by arrow is $f_c = 35$ Hz, hence $\tau_{m.e.p.c.} = 4.54$ ms. The zero frequency asymptote is three times smaller for the spectrum of m.e.p.cs treated with TC indicating that the ratio: curarized m.e.p.c. amplitude/normal m.e.p.c. amplitude, $\alpha = 0.58$, where the ratio of the amplitudes, α , before and after TC is obtained from the ratio of the square roots of the respective spectral densities. Hence, from the relationship, $p = (\tau - \tau_{TC})/(\tau - \alpha\tau_{TC})$, $p = 0.41$ for this particular case.

Table 1 Proportion of the ACh packet (p) attaching to receptors: *a*, for normal endplates by comparison of normal with myasthenic m.e.p.cs; *b*, for a normal endplate by curarizing normal m.e.p.cs; *c*, for a myasthenia gravis endplate by α -bungarotoxin treating MG m.e.p.cs

	$\tau_{m.e.p.c.}$ (ms)	$\tau_{m.e.p.c.}$ (ms)	V_m (mV)	α^*	p
	(normal)	(neostigmine)			
<i>a</i> Normal	1.77 ± 0.07 ($n = 15$)	5.56 ± 0.25 ($n = 16$)	-80	0.35	0.45
Myasthenia gravis	1.80 ± 0.05 ($n = 18$)	3.65 ± 0.12 ($n = 20$)	-80		
	$\tau_{m.e.p.c.}$ (ms)	$\tau_{m.e.p.c.}$ (ms)	V_m (mV)	$\alpha^\dagger$	p
	(neostigmine)	(neostigmine + TC or α -Btx)			
<i>b</i> Normal	5.3	4.0 (TC)	-80	0.78	0.59
<i>c</i> Myasthenia gravis	6.12	5.40 (α -Btx)	-120	0.36	0.17

Values in *a* are mean $\pm$ s.e., where n = no. of endplates examined. All measurements were made at 22°C .

* α = MG m.e.p.c. amplitude/normal m.e.p.c. amplitude.

† α = treated m.e.p.c. amplitude/untreated m.e.p.c. amplitude.

We thank Professor B. Katz for helpful discussions, Mr J. R. Belcher and Dr J. Newsom Davis for help in obtaining biopsies, the patients for their collaboration and the MRC and the Muscular Dystrophy Association of the UK for support.

Received 27 March; accepted 2 June 1980.

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Erythroid developmental agglutinin is a protein lectin mediating specific cell-cell adhesion between differentiating rabbit erythroblasts

F. Lynne Harrison & C. James Chesterton

Department of Biochemistry, King's College London, Strand, London WC2R 2LS, UK

In many developmental systems β -galactoside-specific protein lectins have been identified as components which appear at the cell surface in concert with an initial requirement for cell-cell adhesion during tissue formation^{1–8}. Although some of these lectins have been purified, there has been little direct evidence concerning their role in establishing specific cell-cell contact. A mammalian system where selective cell-cell adhesion is evident and which is amenable to study is that of erythroid differentiation in the adult bone marrow⁹. Here, the differentiation of erythroblasts takes place with the participants clustered together in the vicinity of a macrophage 'nurse' cell until the enucleation stage, when the immature reticulocyte is released and passes through the sinusoidal wall into the circulation. We show here that a small, β -galactoside-specific, protein lectin can be extracted from erythroblast-enriched marrow and purified to homogeneity. This factor, termed erythroid developmental agglutinin (EDA), exhibits properties which strongly suggest that it is responsible for inter-erythroblast recognition and adhesion *in vivo*.

EDA, purified from lactose washes of erythroblast-enriched rabbit bone marrow (see Fig. 1 legend), shows 95–100% Coomassie blue staining in a single component after electrophoresis on native, urea or SDS-containing polyacrylamide gels, where it exhibits a molecular weight of 13,000. No staining is observed with Schiff-periodic acid reagent, and GLC analysis for sugars in EDA hydrolysates detected less than 1 molar equivalent carbohydrate. Completely pure EDA can be obtained by repeating the affinity chromatography step once or twice more. The lectin electrofocuses as a single component at pH 5–6.

The specific location of EDA *in vivo* was demonstrated by immunolabelling. The IgG fraction from anti-EDA antiserum raised in a sheep was absorbed out with that protein from an

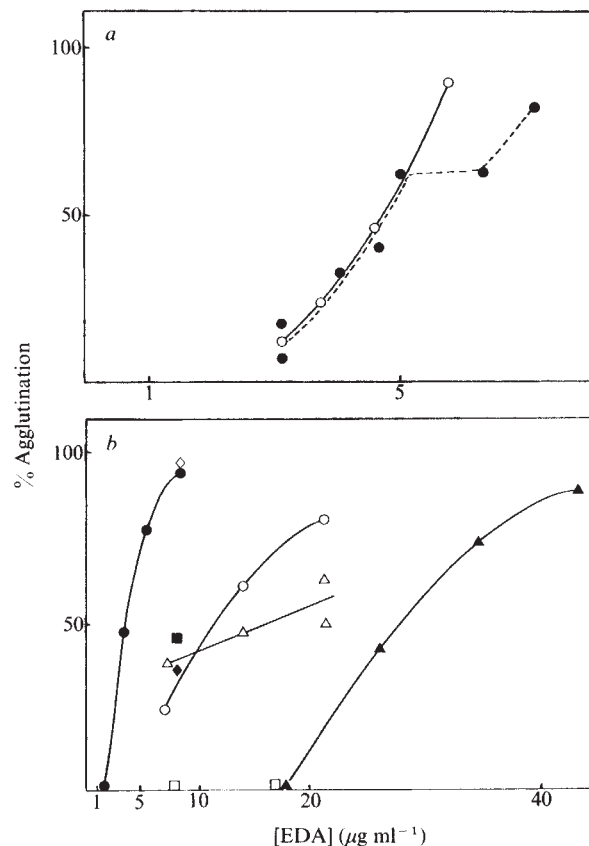


Fig. 1 *In vitro* agglutination studies. The ability of EDA to mediate intercellular cross-linking was tested using cells which were washed twice in MESL and resuspended in buffered medium. In addition, fractions enriched for erythroblasts, immature myeloid cells and marrow reticulocytes were prepared by sedimentation through a Percoll gradient (experimental details will be published elsewhere). The cellular composition of the fractions used was determined by cytocentrifugation and histological staining to be: 90% erythroblast, 5% myeloid, 5% reticulocyte; 69% myeloid, 26% reticulocyte, 5% erythroblast; and 87% reticulocyte, 4% erythroblast, 9% myeloid, respectively. Cell suspensions ($0.25 \text{ ml}, 10^7$ cells per ml buffered medium) were mixed at room temperature by end-over-end rotation (4 r.p.m.) in small plastic vials at various EDA concentrations. Aliquots were taken throughout the incubation, diluted fivefold with 0.2% trypan blue in buffered medium (to check viability) and aggregation was assessed by counting the number of single cells present in a haemocytometer. The points plotted represent single cells lost after 10 min incubation with EDA. In the absence of EDA no agglutination was seen. *a*, Agglutination of a mixed cell population: ●, anaemic rabbit bone marrow; ○, erythroblasts for comparison. *b*, Agglutination of cell fractions: ●, erythroblasts; ○, myeloid cells; △, marrow reticulocytes; □, erythrocytes; ▲, Chinese hamster ovary cells. Erythroblasts in the presence of: ◇, 24 mM sucrose; ■, 0.66 mM lactose; ◆, 0.32 mM thiogalactoside. EDA was purified from the bone marrow of 20 rabbits made anaemic by phenylhydrazine injection¹. The tissue was gently disaggregated and the cells washed $3 \times$ in a total of 30 ml MESL. After centrifugation at 27,000g for 15 min followed by 120,000g for 2 h, the washes were treated with 100% saturated $(\text{NH}_4)_2\text{SO}_4$ and centrifuged at 30,000g for 20 min. Pelleted protein was dissolved in MES (14 mM 2-mercaptoethanol, 2 mM EDTA, pH 8, 150 mM NaCl) and fractionated by gel filtration through Sephadex G-100. Fractions with haemagglutination activity¹⁰ were concentrated by ultrafiltration, applied to a column of Sepharose 4B ($4.4 \times 28 \text{ cm}$) and eluted with MES. EDA was eluted after the main protein peak. Active fractions were concentrated by ultrafiltration.

EDA preparation which did not bind to the affinity column and yielded a single precipitin line in a double-diffusion test against EDA or crude lactose extract. This was used to locate the agglutinin by standard indirect immunofluorescence techniques in whole marrow freshly obtained from healthy or anaemic rabbits. Fluorescence was detected only on the surface of erythroblasts and reticulocytes and this could be completely eliminated by preincubation of the cells and antibody with purified EDA (Fig. 2). Washing the marrow with 0.3 M lactose removes all the cell-surface agglutinin detectable in the same batch of

unwashed marrow. Developing myeloid cells and the macrophage 'nurse' cell were not labelled unless in contact with an erythroblast (Fig. 2).

The agglutinin is capable of linking inert cells together by a mechanism blocked by β -galactosides. The assay used to monitor the lectin during preparation is a simple haemagglutination test with trypsinized rabbit erythrocytes¹⁰. EDA also gives a positive haemagglutination reaction when such cells are fixed in glutaraldehyde, a procedure which chemically cross-links and inactivates surface protein. This activity was inhibited by 0.6 mM lactose and 0.5 mM thiodigalactoside but was not affected by 230 mM sucrose, confirming previous observations on the behaviour of unfixed cells.

The *in vitro* specificity of EDA was first tested on lactose-washed cells from anaemic marrow, a mixed cell population. With increasing concentrations of the lectin, two phases of

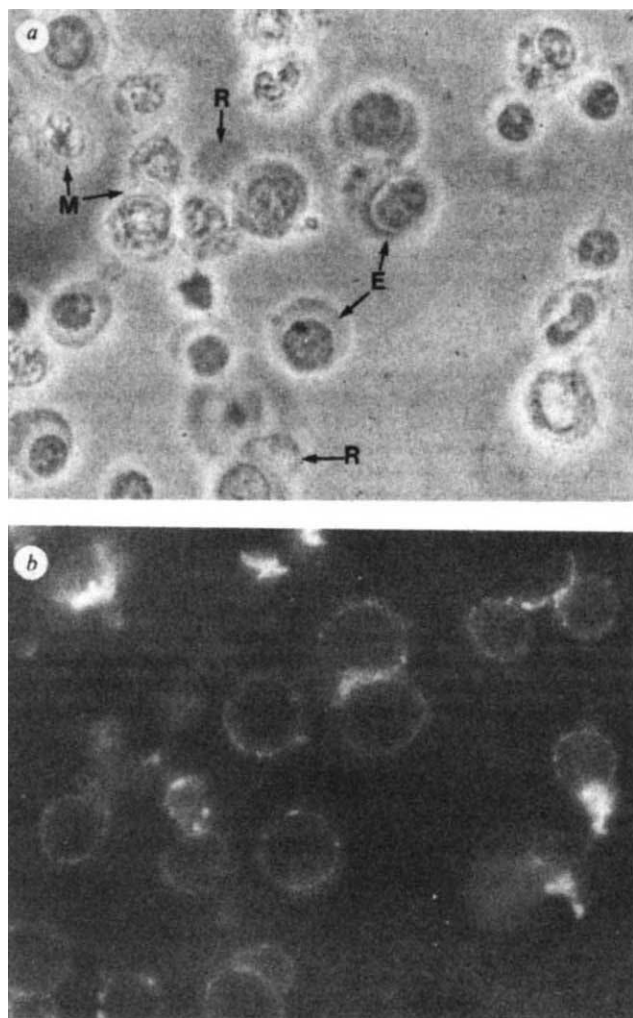


Fig. 2 Detection of EDA in fresh bone marrow by indirect immunofluorescent labelling. The marrow was gently disaggregated in Gibco 109 medium buffered with 25 mM HEPES and the suspension of cells allowed to settle on to microscope slides precoated with polylysine or concanavalin A. The immobilized cells were incubated with preimmune or anti-EDA IgG (3.4 mg ml^{-1}) followed, after washing, by fluorescein-conjugated rabbit anti-sheep IgG. After washing again, the cells were mounted in 80% glycerol in phosphate-buffered saline and viewed with a Vickers M17 microscope using visible light under phase contrast (a) or incident fluorescent illumination (b). Fluorescent labelling was detected strongly on the surface of erythroblasts (E) and weakly on marrow reticulocytes (R). No labelling was detected on the surface of myeloid cells (M). Furthermore, no labelling was seen in the following conditions: when IgG from a preimmune serum was substituted for anti-EDA IgG; if the cells and IgG were preincubated with 0.5 and $1.7 \text{ } \mu\text{g ml}^{-1}$ EDA, respectively; or when the cells were prewashed with MESL (28 mM 2-mercaptoethanol, 2 mM EDTA, pH 8, 150 mM NaCl and 0.3 M lactose). $\times 1,155$.

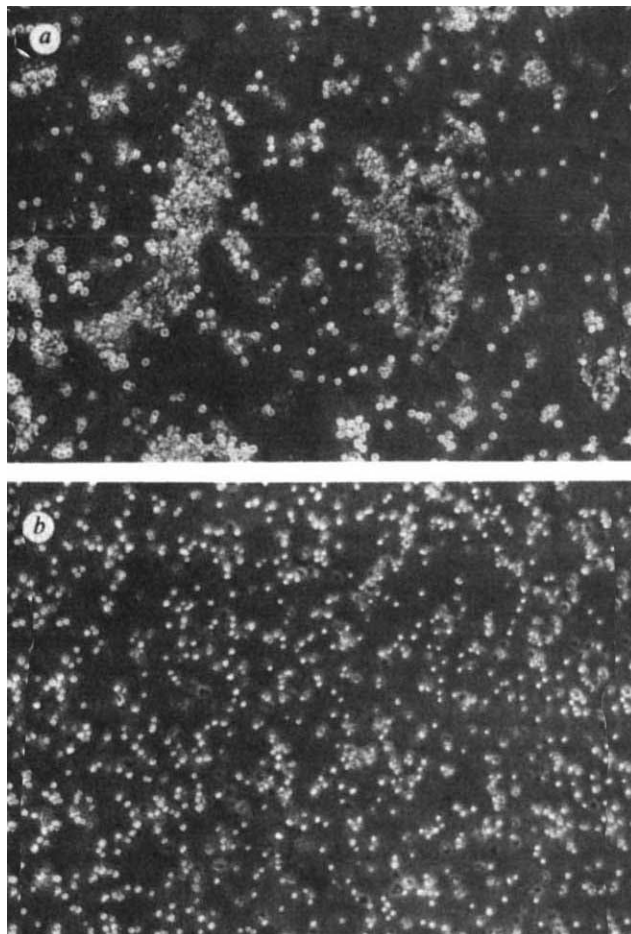


Fig. 3 Effect of anti-EDA Fab on the agglutination of cells bearing cell-surface EDA. Lactose-washed erythroblasts were suspended in buffered medium at $5 \times 10^6 \text{ cells ml}^{-1}$ and allowed to settle out in a glass cavity slide. They were incubated sequentially with $28 \text{ } \mu\text{g ml}^{-1}$ EDA and various concentrations of either anti-EDA Fab or preimmune Fab as described in the text. The cells were resuspended and allowed to settle out once more to allow assessment of agglutination. Fab fragments were prepared by pepsin ($100 \text{ } \mu\text{g ml}^{-1}$) digestion of IgG (10 mg ml^{-1}) for 18 h at 37°C in 70 mM acetate buffer, pH 4, containing 50 mM NaCl. After adjusting the pH to 8 with Tris-base, crude (Fab)₂ was separated from Fc and undigested IgG by gel filtration on G150 and reduced to Fab fragments by incubation with 0.01 M dithiothreitol at 22°C for 30 min followed by 10 min in the presence of 0.022 M iodoacetamide. The product was separated by repeating the gel filtration step and its purity was checked by SDS-polyacrylamide gel electrophoresis. (The method was communicated to us by M. Virji.) The photographs show erythroblasts after incubation with preimmune Fab (a) or anti-EDA Fab (b), both at 5 mg ml^{-1} . $\times 115$.

aggregation were seen. Approximately 60% of the cells agglutinated at $5 \text{ } \mu\text{g ml}^{-1}$ EDA, and the remainder required a higher concentration (Fig. 1a). Phase contrast microscopy showed that the initial aggregates contained almost exclusively erythroblasts which stained preparations revealed constituted 67% of the marrow. The erythroid specificity suggested by these results and previously shown by immunofluorescent labelling was confirmed by the behaviour of different cell fractions in this assay. Lactose-washed erythroblasts from either healthy or anaemic rabbits were agglutinated by $2\text{--}8 \text{ } \mu\text{g ml}^{-1}$ EDA, whereas immature myeloid cells required three or four times more lectin to achieve 50% aggregation, and a Chinese hamster ovary cell line needed a level over fivefold higher (Fig. 1b). Erythroblast agglutination was inhibited by low concentrations of β -galactosides (Fig. 1b). The intra-marrow concentration of EDA is not known, but an estimate can be made from the yield of agglutinin protein after purification. This is approximately 0.2 mg per rabbit or $40 \text{ } \mu\text{g ml}^{-1}$ marrow from anaemic animals and 0.05 mg or $10 \text{ } \mu\text{g ml}^{-1}$ from healthy rabbits; thus, the affinity of

EDA for the erythroblast surface which can be demonstrated *in vitro* seems to be adequate to account for the clustering seen *in vivo*.

The presence of EDA is coincident with the requirement for adhesion *in vivo*. Erythroblasts at all stages of differentiation discernible in the marrow were strongly labelled by anti-EDA IgG but marrow reticulocytes showed a weaker surface fluorescence (Fig. 2). Only faint fluorescence was observed on early circulating reticulocytes and none could be detected on late cells or erythrocytes. Erythroblasts, separated into fractions enriched for the various developmental classes by unit gravity sedimentation¹¹, all showed a similar degree of agglutination by EDA *in vitro*. However, aggregation of bone marrow reticulocytes required higher levels of agglutinin and erythrocytes were not visibly affected apart from a transient clustering at high EDA concentrations (Fig. 1b).

The immunofluorescence studies described above demonstrate that anti-EDA antibody binding does not displace EDA from the erythroblast surface. Therefore, it is possible that if EDA mediated cell aggregation by triggering a secondary mechanism at the cell surface, cell-cell adhesion would not be affected by the binding of an antibody molecule. On the other hand, if an EDA bridging mechanism induces agglutination, the presence of bound anti-EDA IgG, rendered monovalent by conversion to Fab fragments, would almost certainly sterically hinder the process. The experiment was carried out using lactose-washed erythroblasts which had been allowed to settle out into a monolayer on the surface of a cavity slide. The cells adhere to the glass strongly enough for the surrounding medium to be changed without significant disturbance. *In situ*, the erythroblasts were loaded with agglutinin and subsequently incubated with either control preimmune Fab or anti-EDA Fab. After subsequent resuspension, the cells were allowed to settle once more to enable assessment of their state of aggregation (Fig. 3). In the presence of up to 14 mg ml⁻¹ control Fab protein, normal EDA-induced agglutination was observed whereas anti-EDA Fab at 14, 10 or 5 mg ml⁻¹ completely blocked cell clustering; 2.5 mg ml⁻¹ produced an inhibition of about 50%. A second batch of both preparations gave similar results and we find the experiment reproducible with cells from different animals.

Using the experimental advantages of the erythroid system, it has thus been possible to demonstrate that many of the criteria required for identifying molecules which link cells together *in vivo* are fulfilled by EDA. In particular, the fact that this agglutinin will cross-link inert cells and that its action when bound to erythroblasts is blocked by monovalent antibody strongly suggests that a bridging mechanism is involved. EDA is a typical small, acidic, β -galactoside-specific, protein lectin. It may be, therefore, that all such animal lectins act as cell-cell bridging molecules.

Perhaps the temporal role of the β -galactoside-specific lectins in development is now clearer. The inter-erythroblast adhesion mediated by EDA occurs at an early stage of tissue formation and is reversible, the erythroblastic island being a transient structure. Examination of the relationship of other such factors extracted from embryonic tissue to the developmental pattern of the embryo reveals that in all cases activity is only observed at an early stage¹²⁻¹⁵. It is possible that these have a similar role to that of the erythroid lectin. However, in most non-haemopoietic tissue, such links would be overridden by more powerful binding mechanisms. In the marrow, the cohesion is reversed and the maturing cell is lost irretrievably from its tissue of origin.

We thank Tessa Beswick for technical assistance, Susan Godsave, David Wraith and Nicholas Parsons for help with the agglutination experiments and EDA preparation, Dr Elizabeth Hounsell for GLC analysis, Neill Spencer and Karen Wells for analysis of EDA by electrofocusing, Dr N. Totty for Chinese hamster ovary cells, and all in the laboratory for aid in preparing the manuscript. L.H. is a Cancer Research Campaign-supported research fellow and the work was additionally funded from the SRC and MRC.

Received 14 March; accepted 3 June 1980.

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Abelson murine leukaemia virus transformation involves loss of epidermal growth factor-binding sites

Jonas Blomberg*, Fred H. Reynolds Jr†, Wim J. M. Van de Ven† & John R. Stephenson*

* Laboratory of Cellular and Molecular Biology, National Cancer Institute, Frederick, Maryland 21701

† Carcinogenesis Intramural Program, Frederick Cancer Research Center, Frederick, Maryland 21701

Malignant transformation by mammalian RNA sarcoma viruses has previously been shown to involve a reduction in receptor sites for a well characterized 6,000-molecular weight (MW) growth-promoting substance, designated epidermal growth factor (EGF)¹⁻³. Although Abelson murine leukaemia virus (AbLV) resembles sarcoma viruses in its ability to transform embryo fibroblasts in cell culture⁴, AbLV induces a rapid B-cell lymphoid leukaemia rather than fibrosarcomas *in vivo*^{5,6}. The major translational product of AbLV is a highly phosphorylated polypeptide^{7,8} of MW 120,000 which exhibits an associated tyrosine-specific protein kinase activity^{9,10} and probable transforming function¹¹⁻¹⁴. We show here that AbLV transformation resembles transformation by RNA sarcoma viruses with respect to the abolition of EGF-binding sites. EGF binding is restored to control levels following loss of polypeptide expression in morphological revertants of AbLV-transformed clones and remains uninfluenced in cell lines infected with transformation-defective (td) AbLV mutants encoding polypeptides deficient in protein kinase activity. These findings indicate that AbLV transformation involves a polypeptide-associated, tyrosine-specific protein kinase activity which mediates its effect through a mechanism resulting directly or indirectly in the abolition of EGF-binding sites.

The wild-type (wt) and td mutant AbLV FRE Fisher rat and CCL64 mink cell clones analysed in the present study are described in Table 1. In contrast to the wt AbLV-transformed clones, each of the td AbLV mutant clones is highly contact inhibited (data not shown) and lacks the capacity for colony formation in soft agar. Moreover, none of five td AbLV mutant clones were found to be tumorigenic on inoculation into a syngeneic Fisher rat host. Each of the td AbLV non-productively-infected clones express a 120,000-MW polypeptide, P120, at levels comparable with wt AbLV-transformed clones, but in contrast to the latter clones, the polypeptide expressed lacks detectable protein kinase activity. Two morphological revertants of one wt AbLV-transformed mink clone, 64Ab2-2, lack detectable AbLV P120 expression, protein kinase and ability to grow in soft agar. This defect in expression of transformation is cellular rather than viral in nature as demonstrated

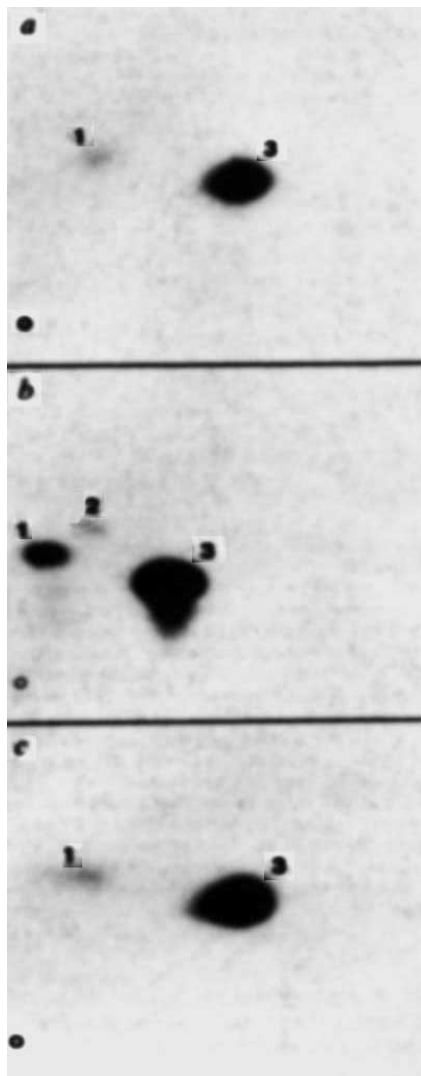


Fig. 1 Identification of amino acid acceptor residues phosphorylated *in vitro* by phosphotransferase activity. *In vitro* ^{32}P -labelled extracts of control FRE cells (a) or FRE cells infected with either wt AbLV (C1 4-4) (b) or td AbLV (2-112-F2) (c) were hydrolysed in 6 M HCl at 100°C for 1 h. Two-dimensional separation was carried out by electrophoresis at 500 V for 3 h in acetic acid/formic acid/water (15:5:80; pH 1.9) (horizontal dimension) and chromatography in 0.5 M ammonium hydroxide/isobutyric acid (3:5) (vertical dimension). *In vitro* phosphorylated amino acid residues were identified by comparing their position relative to phosphoamino acid standards stained with ninhydrin¹⁹. These included phosphothreonine, phosphotyrosine and phosphoserine and co-chromatographed with spots 1, 2 and 3, respectively.

by the presence of AbLV genomic sequences measured by molecular hybridization (data not shown) and the rescuability of wt AbLV by appropriate type C helper viruses^{15,16}.

The above findings do not indicate whether the lack of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ -mediated *in vitro* phosphorylation of AbLV P120 is a defect in the AbLV P120-associated protein kinase activity, or whether it reflects an inability of P120 expressed in td AbLV-infected cells to act as substrate. To resolve these possibilities, we examined the levels of phosphotyrosine in representative wt and td AbLV-infected cell clones. As shown in Fig. 1, phosphotyrosine was not observed to a significant extent in control FRE rat cells but was readily detectable in a representative wt AbLV-transformed cell line. In contrast, appreciable

levels of phosphotyrosine were not observed in td AbLV-infected cell lines. These findings suggest that the lack of phosphorylation of AbLV P120 in td mutant-infected clones does not simply reflect a lack of substrate activity.

In view of the previously reported loss of EGF-binding sites following transformation by avian and mammalian RNA sarcoma viruses¹⁻³, we examined the possibility that AbLV transformation might also result in abolition of EGF-binding sites. For this purpose, each of the above-described wt and td AbLV-transformed cell clones was analysed for the ability to bind ^{125}I -labelled EGF. As summarized in Table 1, EGF-binding by AbLV non-productively-transformed rat and mink cells was ~ 20 -fold lower than that by uninfected control cell lines. Scatchard plot analysis shows this difference to be primarily due to a decrease in receptor site number rather than an altered binding affinity (Fig. 2). Although the absolute number of cellular binding sites on CCL64 mink cells was about seven-fold greater than exhibited by the FRE rat cell line, the binding affinities were similar. Mink or rat cells non-productively

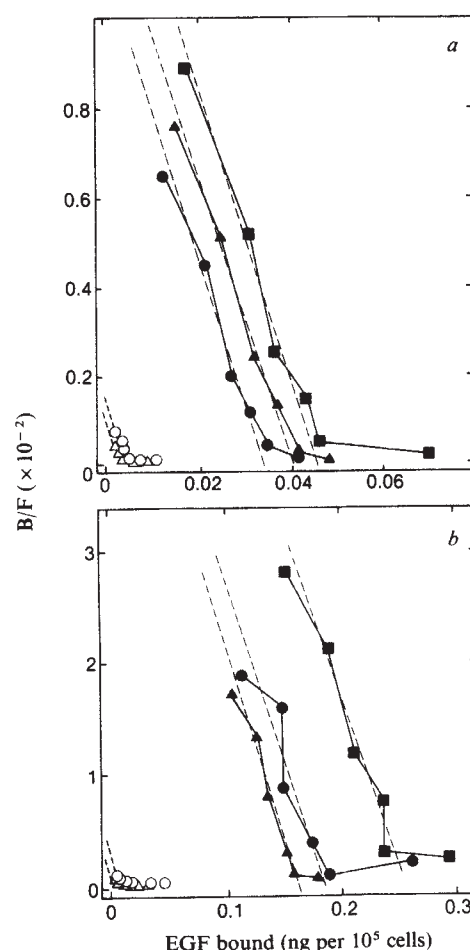


Fig. 2 Analysis of EGF-binding characteristics according to the method of Scatchard²⁵. Mouse EGF (Collaborative Research) was labelled with ^{125}I at high specific activity (10–40 μCi per μg) according to previously published methods^{17,18}. Cell lines were cultured at around 10^5 cells per well in 24-well cluster plates (Costar). After 48 h, individual wells were washed once with complete DMEM, incubated for 3 h at 37°C in serum-free DMEM, and for an additional 4 h at 4°C in 0.2 ml of HEPES-buffered (pH 7.4) DMEM containing 0.1% ovalbumin and concentrations of ^{125}I -labelled EGF ranging from 5,000 to 500,000 c.p.m. Cells were then washed three times in serum-free medium, dissolved in 1% SDS (1 ml) and their radioactivity measured in a γ -scintillation counter. For cell number determinations, duplicate wells were stained with Sedi-stain (Becton-Dickinson). Results are based on triplicate determinations and are expressed as ng EGF bound per 10^5 cells and as the ratio of bound to unbound EGF (B/F). Cell lines tested are described in detail in Table 1 legend and include a, FRE (●), C1 4-4 (○), 2-112-F2 (▲), 2-109-F5 (■), 2-112-RT2 (△), and b, CCL64 (●), 20-1-F6 (○), 64Ab2 (△), 9-104-7 (▲), 64 Ab2-2 (■).

Table 1 Correlation between AblV P120 associated protein kinase activity, EGF binding and malignant transformation

Cell clone		Growth in soft agar (%)	AbLV P120 (μg per mg cell protein)	Protein kinase pmol ³² P incorporated	Phosphotyrosine (% of total cellular phosphoamino acids)	EGF receptor sites	
						No. per cell (×10 ⁻⁴)	Affinity (l mol ⁻¹ × 10 ⁻⁸)
Rat							
Control:	FRE	<0.01	<0.02	<0.01	<0.3	2±0.5	3±1
wt AbLV transformed:	C14-4	16	0.9	0.7	2.8	0.1±0.1	3±2
	2-9-T2	14	1.1	0.5	3.1	0.1±0.1	2±2
td AbLV infected:	34-F2	<0.01	0.8	<0.1	<0.3	8±2	3±1
	2-109-F5	<0.01	1.3	0.03	NT	2±1	3±1
	2-612-F3	<0.01	1.1	<0.01	NT	6±2	3±1
	2-622-F5	<0.01	0.7	<0.01	<0.3	1±1	4±1
	2-112-F2	<0.01	0.8	0.02	NT	4±1	3±1
	2-112-RT2	20	1.1	0.5	1.5	0.1±0.1	3±1
Retransformed td AbLV mutant:							
Mink							
Control:	CCL64	<0.01	<0.02	<0.01	<0.3	15±3	3±1
wt AbLV transformed:	64Ab2	17	1.2	0.3	2.6	0.1±0.1	2±2
	20-1-F6	19	1.8	0.8	2.1	0.2±0.1	2±2
td AbLV infected:	9-104-7	<0.01	0.7	0.02	NT	12±2	4±1
	9-124-2	<0.01	1.3	<0.01	<0.3	8±2	3±1
	9-70-1	<0.01	0.6	<0.01	NT	22±3	3±1
	64-Ab2-2	<0.01	<0.02	<0.01	<0.3	27±4	2±1
Revertant:	64Ab2-56	<0.01	<0.02	0.01	NT	13±2	2±1

Cells were propagated in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% calf serum (Colorado Serum Co.). Details of the isolation and preliminary characterization of each of these cell lines have been previously reported^{16,17}. Plating efficiency in soft agar was determined as previously described^{16,17}. Results represent mean values from three separate determinations. Levels of AblV P120 expression were estimated on the basis of competitive immunoassays for their p12 structural components^{16,17}. *In vitro* phosphotransferase assays were carried out as described previously⁹. Cells (1×10^7) were disrupted in 5 ml of 10 mM Tris-HCl buffer (pH 7.2) containing 100 mM NaCl, 5 mM MgCl₂, 0.1% Triton X-100, by repeated aspiration through a 19-gauge needle. Extracts were preabsorbed overnight with normal goat serum (50 μl) and 0.5 ml of a 10% v/v suspension of protein-A-Sepharose (Pharmacia), and clarified by centrifugation of 40,000g for 90 min. Immunoprecipitation was by addition of 5 μl goat anti-Moloney-MuLV and 50 μl protein-A-Sepharose (10% v/v suspension) to 1 ml extract, agitation overnight and collection of immunocomplexes by centrifugation at 2,000g. Protein kinase reactions were carried out by resuspension of immunoprecipitates or aliquots of total lysates in Tris-HCl buffer (pH 7.2) containing 5 mM MgCl₂, 1 μCi [γ - ^{32}P]ATP 100 Ci mmol⁻¹ (NEN) and incubation for 10 min at 30 °C. Samples were washed in ice-cold 10 mM sodium phosphate buffer (pH 7.2), 0.9% NaCl, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS, collected by centrifugation at 2,000g for 10 min, transferred and collected on glass fibre filters (GF/C, Whatman) with 10% trichloroacetic acid containing 1 mM AMP. Incorporated radioactivity was quantified by liquid scintillation and results expressed as pmol ^{32}P incorporated into trichloroacetic acid-insoluble precipitates and corrected for nonspecific radioactivity at zero reaction time. Determination of phosphotyrosine as a percentage of total cellular phosphoamino acids was determined by scraping individual ^{32}P labelled phosphoamino acids from TLC plates; radioactivity was quantified by liquid scintillation. Results are expressed as the ratio of ^{32}P -labelled phosphotyrosine to total ^{32}P label in all three phosphoamino acids. NT, Not tested. The number of EGF-binding sites per cell and binding affinities are estimated on the basis of Scatchard binding plots prepared according to the method of Scatchard²⁵ and illustrated in Fig. 2. Results represent average values of two to four separate determinations $\pm$ s.e.m. As recently discussed by Norby *et al.*²⁶, values obtained by this means may represent slight overestimations of the actual binding site numbers.

infected with each of eight td AblV mutants examined, exhibited similar numbers of EGF-binding sites and binding affinities to the uninfected control cell lines from which they were derived. Moreover, as shown in Table 1, restoration of AblV P120-associated protein kinase activity following retransformation of a representative td AblV mutant clone by wt AblV was associated with a reduction in the number of EGF receptors. Thus, the defect in transforming potential by td AblV mutants is associated with simultaneous loss of protein kinase activity and an impairment in the process resulting in EGF-receptor site abolition.

As described above, morphological reversion of AblV transformation is associated with loss of detectable levels of AblV P120 expression. Thus, if AblV P120-associated protein kinase activity is necessary for the abolition of EGF-receptor binding sites in AblV-transformed cells, we would predict that such sites would reappear and that cellular phosphotyrosine levels would be reduced to those characteristic of nontransformed cells in revertant clones. To test this possibility, several morphological revertants of AblV-transformed mink clones were analysed with respect to EGF-binding properties and levels of cellular phosphotyrosine. These clones are of particular interest because the transformation defect seems to be cellular in nature and to occur at high frequency^{15,16}. As shown in Table 1, in both of the revertant cell clones examined, phosphotyrosine levels were reduced to control levels. Moreover, in both cell lines, EGF binding was restored to levels comparable to that exhibited by uninfected control cells.

To test the generality of restoration of EGF binding after morphological reversion of transformation, levels of EGF binding by uninfected control, Gardner feline sarcoma virus (FeSV)-transformed cells and morphological revertants of FeSV-transformed mink cells were examined. The Gardner FeSV transformation system was chosen because of its resemblance to AblV in that FeSV has also been shown to encode a high

molecular weight phosphorylated polypeptide translational product^{17,18} with associated protein kinase activity⁹. In fact, we have more recently shown that this activity also has specificity for phosphotyrosine acceptor sites and have demonstrated elevated levels of phosphotyrosine in mink and rat cells following FeSV transformation¹⁹. As shown in Table 2, EGF binding was over 10-fold lower in FeSV-transformed than uninfected control mink cells. Following reversion of one such clone to a nontransformed phenotype, EGF binding was restored to control levels. Thus, an undefined mechanism resulting in simultaneous loss of expression of viral polypeptide expression and phenotypic transformation also leads to restoration of EGF-binding capacity.

The present findings establish an association between AblV-induced transformation and reduction of EGF-binding sites and suggest that both depend on the presence of tyrosine-specific protein kinase, either intrinsic to, or associated with, AblV P120. The fact that EGF binding by td AblV mutant-infected

Table 2 EGF binding by uninfected control, FeSV-transformed and morphological revertant cell clones

Cell clone		EGF receptor sites	
		No. per cell ($\times 10^{-4}$)	Affinity $1 \text{ mol}^{-1} \times 10^{-8}$
Rat			
Control:	FRE	2 ± 0.5	3 ± 1
FeSV transformed:	FeSV-NPC14	0.1 ± 0.1	2 ± 1
Mink			
Control:	CCL64	15 ± 3	3 ± 1
FeSV transformed:	64-FeSV-14	0.3 ± 1	2 ± 1
Revertant:	64-FeSV-14-1	12 ± 4	3 ± 1

The isolation and preliminary characterization of these cell lines have been previously reported^{14,16}. EGF receptor site numbers and binding affinities were determined as described in Table 1 legend.

cell lines is equal to that exhibited by uninfected control cells, despite levels of AbLV P120 expression comparable to those in wt AbLV transformants, suggests that if AbLV does directly interact with EGF-binding sites, its expression in the absence of protein kinase activity is not sufficient for transformation. For instance, phosphorylation of one or more tyrosine acceptor sites within the EGF receptor, AbLV P120 itself, or in some undefined cellular regulatory protein may be involved in the process. Alternatively, reduction of EGF binding associated with AbLV P120 protein kinase activity may be due to activation of one or more cellular growth-promoting factor(s) analogous to sarcoma growth factor (SGF) described by Todaro and colleagues²⁰.

Our results do not resolve the problem of whether the reduced EGF-binding activity associated with AbLV transformation is directly attributable to AbLV P120 protein kinase activity or whether loss of EGF binding represents a secondary phenomenon resulting from expression of the transformed phenotype. The previous demonstration, however, that EGF binding is reduced only partially in avian sarcoma virus-transformed cells and remains essentially unaltered in cells morphologically transformed by DNA viruses such as SV40 and polyoma, argues against the latter possibility. This difference in effect on EGF receptors by RNA and DNA tumour viruses of mammalian origin is somewhat surprising in view of results indicating that transformation by SV40 and polyoma may also involve protein kinase activities²¹⁻²⁴.

Activation of growth factor regulatory systems may represent one of several mechanisms for spontaneous and chemically and virally induced malignant transformation. In particular, the transforming function associated with acquired cellular sequences represented within recombinant genomes of certain RNA tumour viruses, such as AbLV, may be mediated by this pathway. A major advantage of the AbLV system for studies of the role of the growth-promoting factors in transformation relates to the fact that an AbLV-encoded protein with tyrosine-specific protein kinase activity and probable transforming function has been identified. In addition, many td AbLV mutants and morphological revertants have been isolated and characterized in some detail. AbLV transformation thus provides an excellent model system for further elucidation of the molecular events involved in malignant transformation.

We thank K. A. Seese and G. T. Blennerhassett for technical assistance. This work was supported by NCI contract NOI-CO-75380.

Received 14 February; accepted 5 June 1980.

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'Activation-labile' glucocorticoid-receptor complexes of a steroid-resistant variant of CEM-C7 human lymphoid cells

Thomas J. Schmidt*, Jeffrey M. Harmon & E. Brad Thompson

Laboratory of Biochemistry, DCBD National Cancer Institute, NIH Bethesda, Maryland 20205

For cytoplasmic glucocorticoid-receptor complexes to enter and accumulate in the nucleus a temperature-dependent event, 'activation' is required¹⁻⁴. Activation can be achieved *in vitro* by increased ionic strength³⁻⁶, dilution or gel filtration⁷ and is manifested by an increased affinity of steroid-receptor complex for DNA^{3,5,7,8} and an altered elution profile from ion-exchange resins⁹⁻¹¹. Munck and Foley have shown¹² that activated complexes isolated from thymocytes elute from DEAE-cellulose in a manner identical to complexes activated *in vitro*. We report here that DEAE-cellulose chromatography of steroid-receptor complexes from CEM-C7, a cloned human leukaemic T-cell line sensitive to the cytolytic action of glucocorticoids¹³⁻¹⁵, and its steroid-resistant subclone 4R4 demonstrated that steroid receptors of clone 4R4 cannot form stable activated complexes. This defines a new defect in receptor action, activation lability (r^+act^1), which is unlike either the r^- , r^+nt^- , or r^+nt^1 phenotypes previously described for mouse lymphoid variants¹⁶⁻¹⁹.

Both the glucocorticoid-sensitive clone (C7) and the steroid-resistant subclone (4R4) contain specific, high-affinity binding sites for ³H-dexamethasone, although the 4R4 sites display a somewhat lower affinity for dexamethasone than do those of its sensitive parent (Table 1). The source of this difference, which in itself cannot account for the total resistance of 4R4 to 10^{-6} M dexamethasone, may be clonal variation, or it may actually reflect subtle differences in the receptors. Since subsequent experiments indicate that glucocorticoid-receptor complexes of 4R4 are labile in the conditions of the whole cell binding assay (37 °C), the higher K_d obtained may result from non-equilibrium conditions. In fact, binding data obtained using cytosol assays performed at 0-4 °C suggest that both C7 and 4R4 receptors have the same high affinity (3×10^{-8} M) for dexamethasone (data not shown). A more striking difference in the properties of the receptor complexes of the two clones is their relative ability to translocate to the nucleus in physiological conditions (Table 1). No glucocorticoid-receptor complex was detected in the nucleus of 4R4 after incubation for 1 h at 37 °C in the presence of 5×10^{-8} M ³H-dexamethasone, although 40% of the receptor complexes of clone C7 were detected in the nucleus in identical conditions. In contrast to the r^- mouse variants which generally display decreased but detectable nuclear accumulation of receptor complex (40% of the sensitive S49 wild type)^{16,19}, clone 4R4 exhibits no nuclear transfer.

Since glucocorticoid-receptor complexes of clone 4R4 could not be detected in the nucleus we examined the possibility that these receptor complexes were either unable to be activated (r^+act^-) or were labile (r^+act^1) in 'activating' conditions. DEAE-cellulose chromatography of glucocorticoid-receptor complexes from several cell types resolves these complexes into two forms: a high salt-eluting form which does not bind to DNA, nuclei or chromatin and probably represents the 'unactivated' form of the complex, and a low salt-eluting form which does bind to DNA, nuclei and chromatin, and which has been suggested to

* Present address: Fels Research Institute, Temple University School of Medicine, Philadelphia, Pennsylvania 19140.

† To whom all correspondence should be addressed.

represent the 'activated' form of the glucocorticoid-receptor complex^{11,12}. Figure 1a depicts the time course of activation of C7 cytoplasmic receptors which were pre-incubated for 2 h at 0–4 °C with 5×10^{-8} M [1,2,4(*n*)-³H]triamcinolone acetone (³H-TA) and then activated at 20 °C in the presence of 0.2 M KCl for 0–30 min. Immediately after the 2 h incubation and removal of unbound ³H-TA by gel filtration, most receptor complexes were eluted from DEAE-cellulose by 0.22 M potassium phosphate, that is in the 'unactivated' form (peak II). After activation for 15 min or 30 min there was a progressive shift of specifically bound ³H-TA to a form which was eluted at 0.05 M potassium phosphate (peak I), so that after 30 min 50% of the total bound radioactivity was found in peak I with a proportional loss from peak II. The salt concentrations at which peak II and peak I were eluted are essentially the same as those for cytoplasmic receptor complexes detected in rat liver, HTC cells, LA9 cells, rat brain and adrenalectomized rat thymus (Y. Sakaue and E.B.T., unpublished data), and only activated receptor complexes from CEM cells bind to DNA-cellulose²³.

Figure 1b depicts the time course of attempted activation of 4R4 cytoplasmic receptors. Again the glucocorticoid-receptor complexes in the unactivated cytosol were eluted predominantly in peak II (0.21 M potassium phosphate). After activation for

Table 1 Characteristics of binding of ³H-dexamethasone by sensitive (C7) and resistant (4R4) CEM cells

Clone	Binding (sites per cell)*	K_d *	% Nuclear translocation*
C7	20,000	1.2×10^{-8} M	40
4R4	5,900	7.3×10^{-8} M	0

Cells were grown in suspension in RPMI 1640 (NIH Media Unit) containing 10% heat-inactivated fetal calf serum (North American Biologicals) at 37 °C in a humidified atmosphere of 95% air, 5% CO₂. Clone 4R4 is one of 54 spontaneously derived steroid-resistant subclones of CEM-C7 selected in 10^{-6} M dexamethasone¹⁵. For the whole-cell binding assay cells were collected in mid-logarithmic phase and resuspended in Tricine-buffered RPMI 1640 at approximately 10^7 cells per ml. Aliquots (980 µl) of the cell suspensions were added to a series of borosilicate culture tubes (Fisher Scientific) which contained 10 µl of various concentrations of [1,2(*n*)-³H]dexamethasone (38 Ci mmol⁻¹; Amersham/Searle) in 95% ethanol plus either 10 µl of ethanol (uncompeted) or 10 µl of 5×10^{-3} M nonradioactive dexamethasone in ethanol (competed). Cell suspensions were incubated at 37 °C for 1 h. Following the incubation, 3 ml Hanks' balanced salt solution (BSS) at 22 °C were added to each tube, and the tubes were then centrifuged for 1 min at 1,200 g in a clinical centrifuge. The cell pellets were then washed three times in 3 ml of BSS at 22 °C. Final cell pellets were resuspended in 1.6 ml BSS and 1.0 ml was added to 10 ml Aquasol liquid scintillation cocktail (NEN) and radioactivity was determined in a Beckman LS 9000 counter. A 0.2-ml aliquot of the cell suspension was counted (Coulter Model ZB) to determine final cell recovery. The difference between the total d.p.m. and the noncompeting d.p.m. represents specifically bound ³H-dexamethasone and was expressed as sites per cell. K_d values and sites per cell were determined by analysing the data by the method of Scatchard²⁰. For determination of nuclear translocation a minor modification of the procedure of Munck and Wira²¹ was utilized.

* Values represent means of at least two determinations.

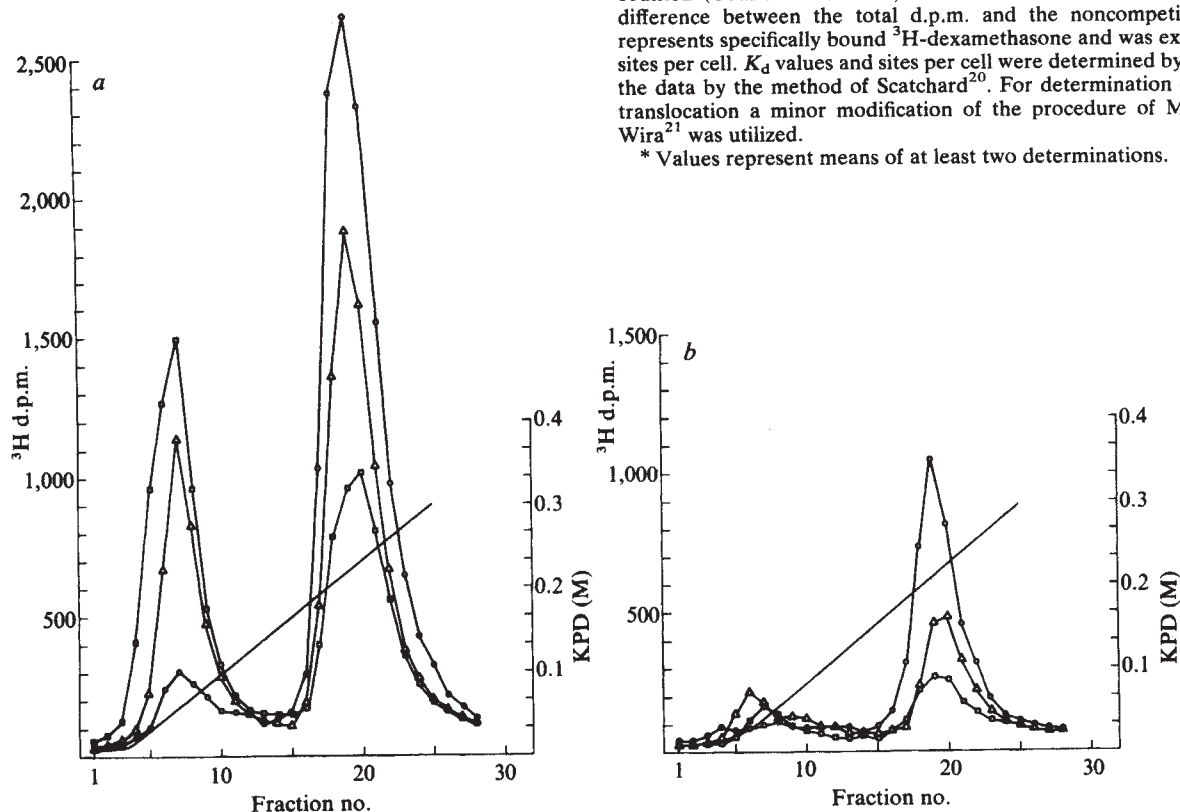


Fig. 1 Time course of activation (20 °C, 0.2 M KCl) of cytoplasmic [1,2,4(*n*)-³H]triamcinolone acetone-receptor complexes of CEM-C7 (a) and 4R4 (b) (○, unactivated control; △, 15 min activation; □, 30 min activation). Cells (1×10^6 cells per ml) were collected, washed once in an excess of ice-cold Hanks' BSS, and homogenized in 5 mM potassium phosphate buffer containing 0.5 mM dithiothreitol, pH 7.3 (KPD) at 4 °C with a Ten Broeck ground glass homogenizer. Cytosols were isolated after centrifugation for 1 h at 100,000g in a Spinco Model L refrigerated centrifuge. Cytosolic protein concentrations were determined by the method of Lowry *et al.*²² and were 6 to 9 mg ml⁻¹. Cytosols were incubated for 2 h at 0–4 °C with approximately 5×10^{-8} M [1,2,4(*n*)-³H]triamcinolone acetone (29 Ci mmol⁻¹; Amersham/Searle) before activation. Following activation (20 °C, 0.2 M KCl) free steroid and salt were removed by passage over G25 columns (PD 10; Pharmacia) pre-equilibrated with 5 mM KPD buffer. 700 µl of the post-G25 cytosol were then loaded on to a DEAE-cellulose (DE52; Whatman) column (3 ml bed volume) which had been pre-equilibrated with 5 mM KPD. Bound radioactivity was then eluted by a linear 5 mM to 400 mM KPD gradient and 1.0-ml fractions collected. All chromatographic procedures were performed at 4 °C. Aliquots (400 µl) of each fraction were added to 10 ml of Aquasol and assayed for radioactivity. Salt concentrations were measured with a Radiometer electroconductivity meter. In similar experiments cytosols incubated with a 1,000-fold excess of nonradioactive TA failed to reveal any ³H-TA bound to DEAE-cellulose columns. Since these lymphoid cells are devoid of progesterone receptors (J.M.H. and T.J.S., unpublished data) the binding of ³H-TA can be assumed to be to glucocorticoid receptors.

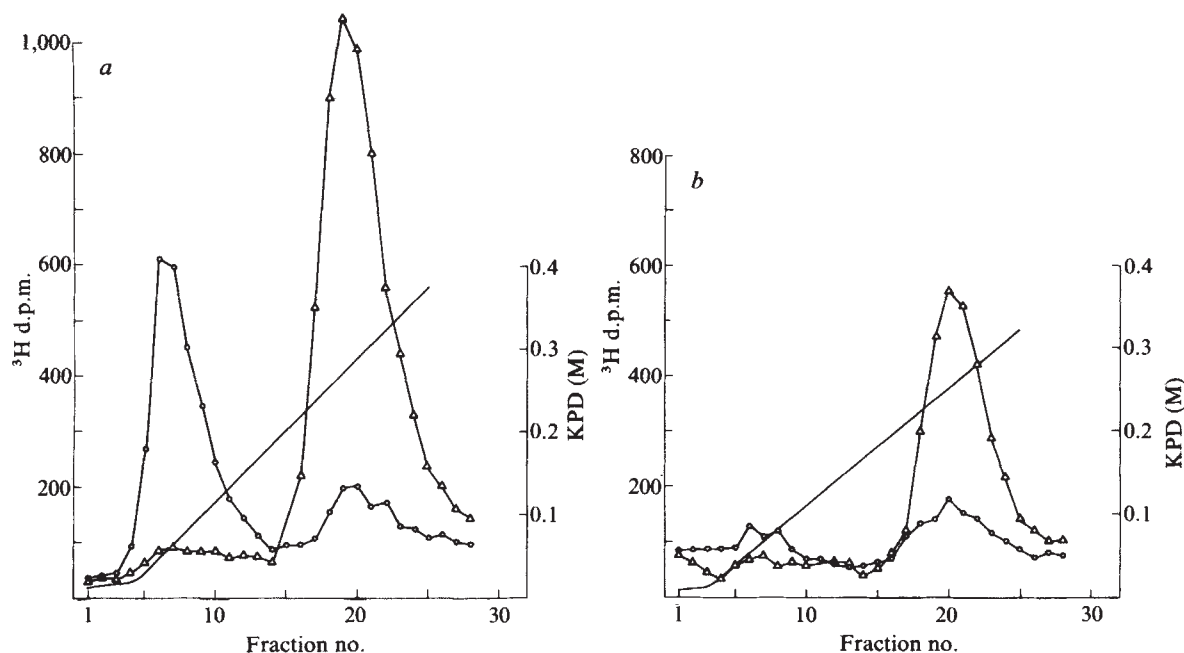


Fig. 2 DEAE-cellulose chromatograms depicting activation (30 min at 20°C, 0.2 M KCl) of cytoplasmic [1,2,4(n)-³H]triamcinolone acetonide-receptor complexes of CEM-C7 (a) and 4R4 (b) in the absence (○) and presence (△) of molybdate. Cell cytosols were prepared, incubated with ³H-TA, and activated as in Fig. 1 with the exception that half of each cytosol was activated in the presence of 50 mM molybdate.

15 min there was a significant depletion in the radioactivity associated with peak II as seen for the wild type, but only a very small increase in the radioactivity associated with peak I (0.05 M potassium phosphate). Continued exposure to activation conditions for 30 min resulted in a further reduction of peak II with no further increase in the small amount of bound radioactivity eluted in peak I so that the total bound radioactivity was reduced by approximately 50%. These data clearly suggest that glucocorticoid-receptor complexes from 4R4 are labile in activation conditions. The small increase in peak I seen in the time course of activation of 4R4 receptor complexes after 15 min (Fig. 1b) suggests that at least some activation is possible. The subsequent disappearance of this peak after prolonged (30 min) activation suggests that it may be the activated form of the receptor complex which is labile. This instability could result from actual denaturation of the receptor protein or from dissociation of the steroid from receptor.

Sodium molybdate (Na₂MoO₄) inhibits the activation of glucocorticoid-receptor from rat liver, rat thymus, mouse L cells and human leukaemic cells (T.J.S., unpublished data)^{24,25}. It also inhibits the activation of avian oviduct progesterone receptors^{26,27}. When 50 mM molybdate was incubated with C7 cytosol receptors during activation, the characteristic shift of steroid-receptor complex from peak II to peak I was not observed (Fig. 2a), demonstrating the ability of this compound to inhibit the activation of human lymphoid receptors. Attempted activation of 4R4 steroid-receptor complexes in the presence of 50 mM molybdate produced the result shown in Fig. 2b. In contrast to the loss of binding in the absence of molybdate, all of the binding was retained and eluted in peak II. This retention of binding most probably reflects the failure to generate a labile activated form of the 4R4 steroid-receptor complex in the presence of the inhibitor of activation, molybdate. Alternatively molybdate may have another, as yet undefined effect, whereby it is capable of stabilizing an otherwise unstable 'unactivated' form of the steroid-receptor complex.

Mouse lymphoid variants of S49 and W7, resistant to the cytolytic action of glucocorticoids, either lack receptor (r⁻) or have receptor with diminished ability to enter the nucleus with

reduced affinity for DNA-cellulose (r^{nt}) or receptors which paradoxically bind more avidly to DNA cellulose (r^{nt})¹⁶⁻¹⁹. In the case of the r^{nt} and r^{nt} phenotypes it is presumed that activation is normal since it is the affinity of steroid-receptor complexes for DNA and not their ability to bind which is altered. We have identified a new phenotype, r^{act}, in a steroid-resistant variant of a human leukaemic cell line. DEAE-cellulose chromatography of the glucocorticoid-receptor complexes of this resistant variant failed to demonstrate the formation of stable activated complexes. This result explains our failure to detect ³H-dexamethasone-receptor complexes in 4R4 nuclei in physiological conditions and indicates an additional mechanism for the acquisition of steroid resistance. These results suggest that the predominant mechanism(s) of steroid resistance in human and mouse lymphoid cell lines may differ and the steroid-resistant variants of CEM-C7 may provide a more useful model system for the study of glucocorticoid resistance in human T-cell leukaemia.

This research was supported in part by an NIH Postdoctoral Fellowship (1F32 CA05447-02) awarded by the NCI to T.J.S. We thank Mrs Jean Regan for editorial assistance.

Received 16 November 1979; accepted 9 May 1980.

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Insertion of *E. coli* photoreactivating enzyme into V79 hamster cells

Betsy M. Sutherland & S. G. Hausrath

Biology Department, Brookhaven National Laboratory, Upton, New York 11973

Photoreactivating enzyme mediates the specific repair of UV light [220-300 nm, (UV)]-induced cyclobutyl pyrimidine dimers in DNA¹. It binds to dimer-containing DNA, and on absorption of light in the wavelength range 300-500 nm monomerizes the dimer, restoring biological activity to DNA². The specificity of the enzyme for pyrimidine dimers in DNA allows its use as an analytical tool. If UV-induced biological damage is photoreactivable, dimers are probably a major cause of that damage. Thus dimers have been implicated in producing death and mutation in prokaryotes and in simple eukaryotes³. Although this photoreactivation test has great potential value in assessing the role of dimers in UV-induced damage in mammalian cells, its use in cultured mammalian cells has been limited by the dependence of photoreactivating enzyme levels on the cell species⁴, genotype⁵ and culture medium^{6,7}. We have developed a method for insertion of *Escherichia coli* photoreactivating enzyme into mammalian cells, and show here that the inserted bacterial enzyme can mediate photoreactivation of pyrimidine dimers in V79 rodent cells.

We selected V79 Chinese hamster cells as a mammalian cell line with minimal endogenous photoreactivating enzyme (rodent cells contain no more than about 10% as much photoreactivating enzyme as do human cells⁴; also, V79 cells undergo excision repair very slowly⁸⁻¹⁰). Cells were grown in a minimal essential medium known to be unfavourable for photoreactivating enzyme production⁶. We also required an effective insertion agent which did not inactivate the *E. coli* enzyme. Polyethylene glycol (PEG) 6000 proved satisfactory in all these respects.

We first examined dimer monomerization in V79 cells treated with PEG, *E. coli* photoreactivating enzyme and visible light. Cells were grown in 60-mm plastic culture dishes overnight; the medium was removed, the cells were washed twice with 2 ml phosphate-buffered saline (PBS; 0.171 M NaCl, 3.4 mM KCl, 10.1 mM Na₂HPO₄, 1.9 mM KH₂PO₄), and 1 ml PBS was layered over the cells. They were exposed to UV light from a low pressure mercury bulb; UV exposures were measured with a Jagger meter¹¹ calibrated against a Yellow Springs Instrument Company radiometer. All procedures were carried out under Sylvania F40G0 safe lights. After exposure to UV light, the PBS was removed, and the cells treated for 2 min with 3 ml of insertion mixture¹² [prepared by mixing 30 g of PEG 6000, Biological Laboratories (which had been autoclaved for 30 min) with 60 ml of medium without serum at 45 °C. The mixture was then cooled to 37 °C]. The insertion mixture was removed, the cells drained briefly, rinsed with 1 ml PBS and 0.1 ml (1.1 mg) of partially purified photoreactivating enzyme from *E. coli* W 3350 λ *phr* (ref. 13) [fraction III (ref. 14), specific activity ~600 units mg⁻¹] was layered over the cells. The cells were allowed to

stand at room temperature for 15 min, then either exposed to photoreactivating light from a 60-W incandescent bulb, or kept in the dark. Small amounts of PBS (~0.2 ml) were added to the dishes as required to prevent drying of the cells. Cells were collected by scraping into PBS, and centrifugation; their DNAs were extracted, then treated with the *Micrococcus luteus* dimer-specific UV-endonuclease (which makes a single-strand scission adjacent to each dimer)¹⁵, and the relative sizes of the resulting DNAs determined from their electrophoretic mobilities in alkaline agarose^{16,17}.

Figure 1 shows the result of such an experiment. Trace *a* represents DNA from unirradiated cells. Exposure of the cells to 10 J m⁻² of UV radiation (trace *b*) produces UV endonuclease-sensitive sites (dimers) in the DNA, and thus a reduction in the size of the DNA after endonuclease treatment. After UV-irradiated cells were treated with insertion mixture and *E. coli* enzyme, followed by photoreactivating light, almost all of the UV endonuclease-sensitive sites disappeared, implying substantial monomerization of dimers (trace *c*). Treatment of the cells with insertion mixture plus photoreactivating enzyme in the absence of photoreactivating light did not affect the dimer content of the DNA (trace *d*), nor did incubation in the light of

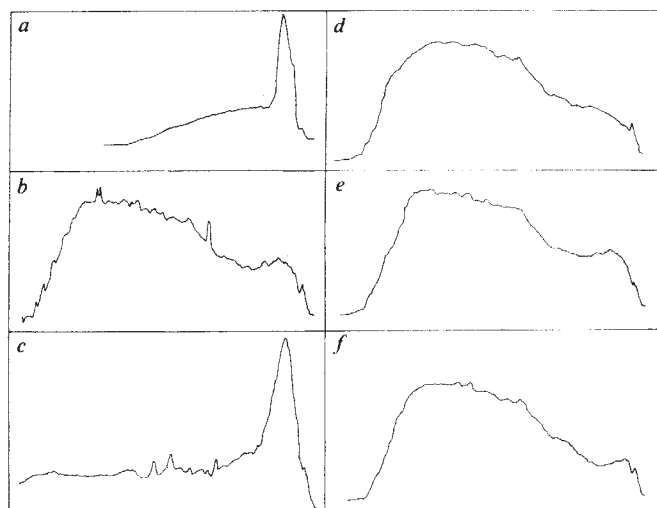


Fig. 1 Alkaline agarose gels of DNA from V79 cells which were: *a*, unirradiated; *b*, exposed to 20 J m⁻² of 254 nm radiation after treatment with PEG and photoreactivating enzyme and killed immediately; *c*, UV irradiated and treated with PEG, photoreactivating enzyme, then 60 min of photoreactivating light; *d*, UV irradiated, treated with PEG, photoreactivating enzyme and kept in the dark for 60 min; *e*, UV irradiated, treated with PEG (but no photoreactivating enzyme) and then 60 min photoreactivating light; *f*, UV irradiated and treated with photoreactivating enzyme (but no PEG) and 60 min photoreactivating light. Cells were collected by scraping into PBS, centrifuging at 5,000 r.p.m. in the SE12 rotor of the Sorvall RC2B for 5 min, resuspension in 0.25 ml buffer A-1 (50 mM Tris, pH 8, 10 mM EDTA, 0.4 M NaCl) and addition of 6 μ l of freshly prepared 5% sodium lauryl sulphate, followed by 0.25 ml of redistilled phenol equilibrated with buffer A-1. The samples were rotated gently for 30 min, centrifuged, the upper layer removed and ethanol-precipitated. The precipitated DNA was centrifuged for 2 min in a Brinkman Microcentrifuge, drained briefly and resuspended in 20 μ l of reaction buffer (30 mM Tris pH 8, 40 mM NaCl, 1 mM EDTA). To samples (10 μ l) of the mixture was added 15 μ g of a *M. luteus* dimer-specific endonuclease preparation¹⁵. The mixture was incubated at 37 °C for 20 min. A stop mixture (2 μ l of 0.5N NaOH, 25% glycerol containing 0.125% bromocresol green) was added, and the mixture applied to a 12 $\times$ 14 cm agarose gel containing 0.29 g agarose, 59 mM NaOH and 4 mM Na₃EDTA. The gels were electrophoresed at 16V for 22 h, neutralized in 500 ml 0.1 M Tris, pH 8, stained in 0.1 M Tris pH 8 containing 0.5 μ g ml⁻¹ ethidium bromide. The gels were photographed using Polaroid type 55 positive/negative film, and the negatives scanned using a Joyce-Loebl densitometer.

Table 1 Fate of dimers in UV irradiated V79 cells

Treatment	% Radioactivity in dimers [(TT+UT) × 10 ² /total]
1 No UV	0.009, 0.005
2 +UV, kill immediately	0.740
3 +UV, 90 min incubation in dark	0.722, 0.682, 0.702, 0.621
4 +UV, 40 min illumination	0.670, 0.650
5 +UV, 90 min illumination	0.562, 0.713
6 No UV, 90 min illumination	0.02

V79 cells were labelled for 24 h in a minimal essential medium containing 10% dialysed fetal calf serum (inactivated at 55 °C for 30 min), and 2 $\mu\text{Ci ml}^{-1}$ ³H-thymidine (specific activity 60 mCi mmol⁻¹). Cells were washed with PBS, exposed to 500 J m⁻² from a germicidal lamp with most of its emission at 254 nm, then killed immediately, kept in the dark or exposed to photoreactivating light from a 100-W incandescent bulb at 20 cm. Cells were collected by scraping into PBS and centrifuging at 15,000 r.p.m. in the Sorvall SE-12 rotor. The DNA was hydrolysed with 88% formic acid, chromatographed on silica gel thin-layer plates in ethyl acetate/H₂O/*n*-propanol (4:2:1)²⁴, sliced and counted in a LKB scintillation counter. Similar determinations for cells exposed to 50 J m⁻² gave no indication of substantial excision or photoreactivation in the times used in the PEG insertion experiments.

cells treated with insertion mixture (but without photoreactivating enzyme) (trace *e*) or incubation in the light of cells treated with enzyme and photoreactivating light (but no PEG) (trace *f*).

As these experiments showed the apparent monomerization of cellular dimers by exogenous *E. coli* photoreactivating enzyme, it was important to show that the cells did not contain endogenous enzyme which might be mediating the reaction. We thus examined cellular photoreactivation and photoreactivating enzyme in three series of experiments. We first labelled the cells with ³H-thymidine, and collected them without irradiation (Table 1, line 1), exposed them to UV (Table 1, line 2), kept them in the dark (line 3) or exposed them to photoreactivating light (lines 4, 5). Dimer photoreactivation in V79 cells grown in minimal essential medium was not detectable after either high (500 J m⁻²) or low (50 J m⁻²) UV exposures. We also examined the loss of UV-endonuclease sensitive sites in V79 cells untreated with insertion mixture or photoreactivating enzyme in experiments similar to those in Fig. 1. Exposure of UV light-irradiated cells to photoreactivating light did not affect the number of endonuclease sensitive sites in the DNA, thus indicating an absence of detectable endogenous photoreactivation. We also tested cell extracts directly for the presence of photoreactivating enzyme activity. No enzyme activity could be detected in standard photoreactivating enzyme assays¹⁸. We thus conclude that it is likely that the disappearance of dimers seen in Fig. 1 is mediated by *E. coli* photoreactivating enzyme inserted into the cells rather than by endogenous V79 enzyme.

This method for inserting *E. coli* photoreactivating enzyme into mammalian cells extends the range of cells in which photoreactivation of pyrimidine dimers can be examined. Several types of cells are especially suited for these studies. For example, rodent cells, in which excision and post-replication repair have been studied extensively¹⁹, contain low levels of endogenous photoreactivating enzyme and have thus not been amenable to photoreactivation studies. In addition many cell strains derived from patients with xeroderma pigmentosum are deficient in photoreactivating enzyme and cellular photoreactivation^{5,20}. Finally, even those mammalian cells which are capable of producing photoreactivating enzyme (presumably *Phr*⁺) must be grown on suitable medium for expression of the *Phr* gene. The development of this method lifts this restriction and should allow examination of photoreactivation in a variety of media. Insertion of an enzyme which can specifically reverse pyrimidine dimers will allow a determination of the role of dimers and of other photoproducts in the induction of cellular damage.

Two other systems have been developed for insertion of exogenous repair enzymes. Smith and Hanawalt studied the action of the phage T4 endonuclease V in isolated nuclei of human cells²¹. Tanaka *et al.* used Sendai virus to insert the T4 endonuclease into intact human cells^{22,23}. They both increased unscheduled DNA synthesis and survival (about a fivefold increase) on addition of Sendai virus plus the endonuclease.

Our attempts to insert *E. coli* photoreactivating enzyme into V79 cells with UV light inactivated Sendai virus were unsuccessful; we thus developed the PEG insertion method [Yarosh (personal communication) has used a similar method for insertion of *M. luteus* UV endonuclease into V79 cells], which has several advantages: (1) PEG is available commercially, and is stable, (2) it is not a potential biohazard and does not yield productive or latent infection of treated cells, and (3) it is gentle to the photoreactivating enzyme, which is inactivated by many reagents. Finally, because the *E. coli* photoreactivating enzyme is larger (monomer molecular weight 35,000; the enzyme also aggregates to form higher multiples) than enzymes inserted by the Sendai method (the molecular weight of the T4 endonuclease is 20,000), it may offer a general method of insertion of enzymes into mammalian cells.

This work was supported by grants and a Research Career Development Award to B.M.S. from the National Cancer Institute of the NIH, and the Department of Energy.

Received 6 March; accepted 27 May 1980.

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Innocuous biological freezing during warming

W. F. Rall*‡, D. S. Reid† & J. Farrant*

* Clinical Research Centre, Watford Road, Harrow HA1 3UJ, UK

† Unilever Research, Colworth House, Sharnbrook, Bedford MK44 1LQ, UK

Intracellular freezing during the cooling of living cells is reported to reduce or abolish their recovery on thawing¹. In microscopical and microprobe studies, low-temperature techniques may induce intracellular ice artefacts². The amount of intracellular ice can be reduced either by selecting a slow cooling procedure that allows extracellular crystallization and cellular dehydration before the intracellular ice forms or by the ultra-rapid cooling of very small samples to minimize the nucleation and growth of ice³. However, a few reports suggest that in some

‡ Present address: ARC Institute of Animal Physiology, Animal Research Station, 307 Huntingdon Road, Cambridge CB3 0JQ, UK.

conditions intracellular ice may be either innocuous⁴ or even sometimes beneficial⁵ for the survival of function. We have therefore used here the technique of low-temperature light microscopy to examine the relationship between intracellular freezing and cell injury. We followed the morphological appearance of eight-cell mouse embryos during cooling and warming in medium containing dimethyl sulphoxide (DMSO; 1.5 M) and correlated it with their functional survival after thawing. Unexpectedly, we found that ice formed in the cytoplasm of embryos during slow warming in conditions when it did not form during cooling. Death of embryos, however, did not correlate with the temperature during slow warming at which intracellular ice appeared (-85°C). The occurrence in some conditions of freezing at these low temperatures during warming has important implications both for the preservation of function of cells and for the preservation of morphology in electron microscopy.

The mouse embryo system was chosen because of the availability of a stringent *in vitro* survival assay (development to expanded blastocyst)⁶ and the ease of observing the large blastomeres during cooling and warming on the low-temperature stage of a light microscope⁷. The embryos were cooled slowly ($\sim 0.5^{\circ}\text{C min}^{-1}$) to -40°C and then rapidly to $< -150^{\circ}\text{C}$. In these conditions, they exhibit high survival after rapid warming ($\sim 500^{\circ}\text{C min}^{-1}$) but are dead after slow warming ($< 20^{\circ}\text{C min}^{-1}$). Injury following slow warming is believed to be associated with the migratory recrystallization of intracellular ice that formed during rapid cooling⁸.

Figure 1 shows the typical microscopical appearance of two eight-cell embryos in DMSO (1.5 M) during cooling and warming. The formation of intracellular ice is usually characterized by the sudden blackening or 'flashing' of the cytoplasm when viewed using phase contrast optics⁹. This blackening is believed to result from the scattering of light by the surfaces of ice crystals and possibly gas bubbles¹⁰. During the slow cooling of the embryos to -40°C (Fig. 1a–1f), extracellular freezing occurred and the blastomeres shrank as reported for other cells^{11–13}. During the subsequent rapid cooling to $\sim -150^{\circ}\text{C}$ (Fig. 1g), the appearance of the embryos did not change, indicating the absence of visible intracellular ice. However, when the embryos were then warmed slowly, they 'flashed' (Fig. 1j), suggesting the formation of intracellular ice when the temperature reached -85°C . On continued slow warming, a complex series of phenomena was observed. Changes included: the gradual appearance (-85 to -70°C ; Fig. 1j–l) and disappearance (-70 to -40°C ; Fig. 1l–q) of a dark crystalline material outside the zona pellucida and perhaps within the perivitelline space, an abrupt increase in the mobility of this material at -70°C (not visible in Fig. 1, but discernible in videotape recordings), the sudden appearance of the zona pellucida at -65°C (Fig. 1m), a second darkening of the cytoplasm at -55°C (Fig. 1o) and finally, the disappearance of the dark intracellular material at -40°C . After thawing, the embryos appeared morphologically abnormal (Fig. 1r). When similarly cooled embryos were warmed rapidly ($\sim 250^{\circ}\text{C min}^{-1}$) from -120°C or below, both videotape and visual observations revealed none of these changes in appearance during warming. Furthermore, if slow warming was converted to rapid warming at $\sim -75^{\circ}\text{C}$, the second darkening of the embryos was not observed. In both these instances, the embryos were morphologically normal after thawing.

The critical temperature range associated with injury during slow warming was found from parallel survival studies. The embryos were cooled slowly to -40°C and then rapidly to -196°C . They were first warmed slowly ($\sim 2^{\circ}\text{C min}^{-1}$) to various temperatures and then rapidly ($\sim 500^{\circ}\text{C min}^{-1}$) to 20°C (Fig. 2). The data demonstrate that the embryos survived slow warming providing conversion to rapid warming was done before the temperature reached -70°C . Slow warming to -60°C or above resulted in the death of the embryos. Functional injury therefore only occurred if slow warming was continued some 15 – 20°C above the temperature (-85°C) at

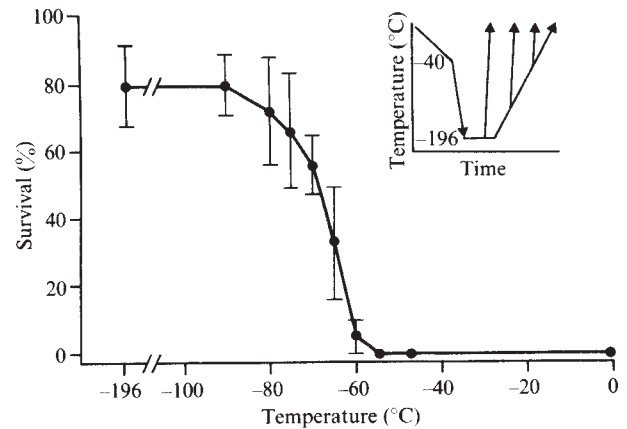
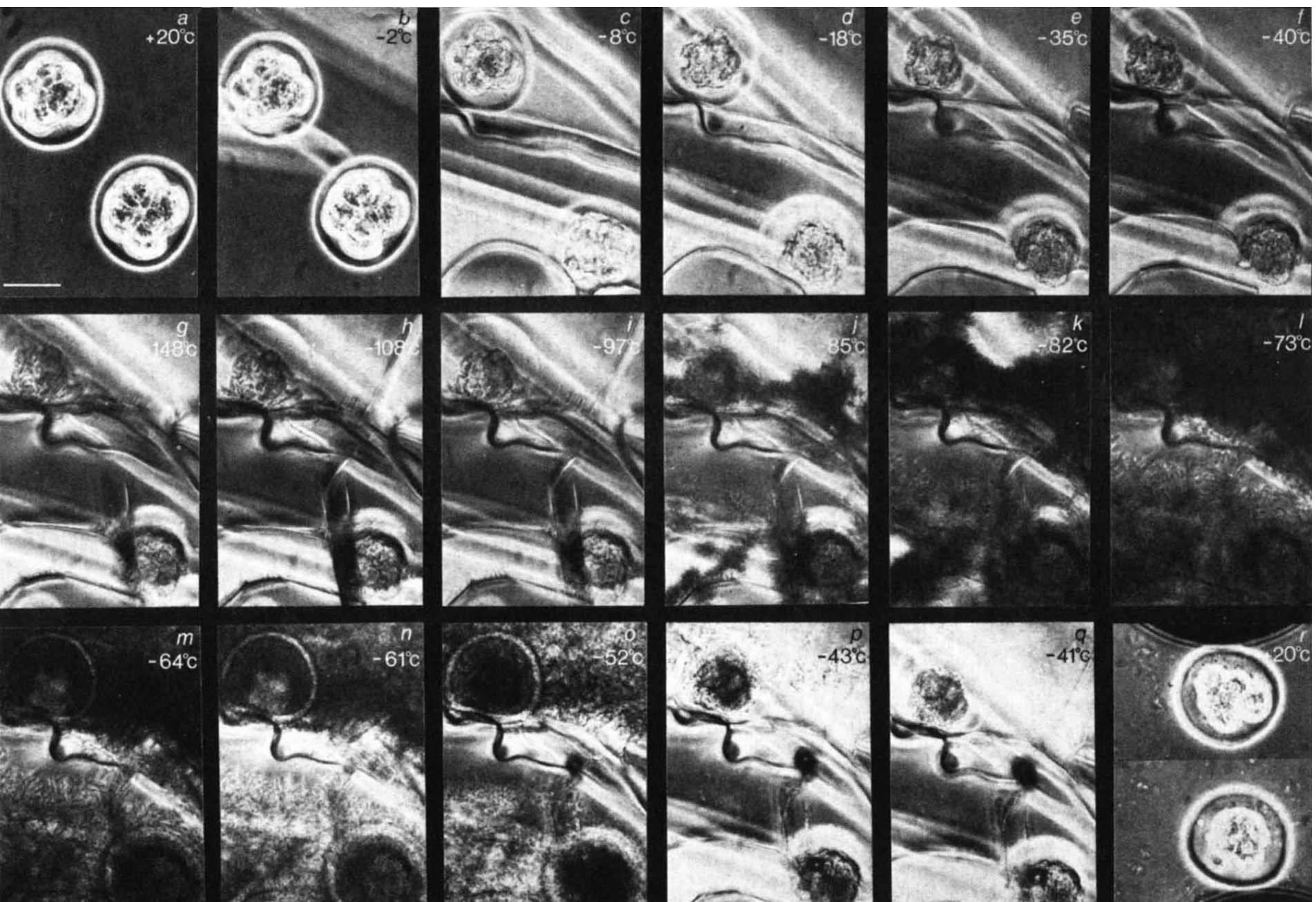


Fig. 1 Microscopical appearance of two eight-cell mouse embryos during cooling and warming. Embryos were flushed from the oviducts of superovulated TO (Crc: TO) or MF1 (Ola: MF1) female mice that had been mated ~ 64 h earlier with TO males. Embryos were collected and handled as described previously¹⁸. The embryos were placed into phosphate-buffered saline (PB1)¹⁹ containing DMSO (1.5 M) for at least 20 min at $\sim 20^{\circ}\text{C}$ to allow complete permeation of the DMSO²⁰. Approximately 10 embryos were then placed into a small drop of the PB1–DMSO solution on a 13-mm diameter coverglass and immediately covered with another coverglass. A hole reinforcer for loose-leaf paper (WEB self-adhesive reinforcements, Westwood Stationery) on one of the coverglasses prevented the embryos from being crushed. The coverglass sandwich was then placed onto the temperature-controlled slide of a low-temperature microscope stage⁷. The appearance of the embryos was recorded on time-lapse videotape and photographed onto Ilford Pan-X film using phase-contrast optics on a Leitz Ortholux II microscope. The embryos were cooled slowly ($\sim 0.5^{\circ}\text{C min}^{-1}$) from $+4$ to -40°C and photographs b–f were taken at the indicated temperatures. Photograph b shows the extracellular ice front as it passes the embryos. The embryos were then cooled rapidly ($\sim 250^{\circ}\text{C min}^{-1}$; photograph g). Subsequently, the embryos were warmed rapidly to -120°C and then slowly ($\sim 2^{\circ}\text{C min}^{-1}$) to ambient temperature (photographs h–r). Scale bar, $\sim 50\text{ }\mu\text{m}$.

Fig. 2 Survival of eight-cell mouse embryos cooled slowly to -40°C and then rapidly to -196°C as a function of the temperature at which slow warming was converted to rapid warming (see insert diagram). Embryos were collected as noted in Fig. 1 legend, and 10–15 embryos were transferred into glass test tubes (10×75 mm) which contained 0.1 ml PB1. The embryos were held at $\sim 20^{\circ}\text{C}$ and an equal volume of PB1 containing DMSO (3.0 M) was added and mixed (final DMSO concentration 1.5 M). The embryos were held at $\sim 20^{\circ}\text{C}$ for 20 min to allow complete permeation of the DMSO²⁰. The samples were then placed into a -7°C bath and 2 min later were nucleated with an ice crystal. After ~ 5 min, the samples were transferred into an alcohol bath at -7°C that was cooling at $\sim 0.5^{\circ}\text{C min}^{-1}$ (ref. 18). When the temperature of the bath reached -40°C , the samples were quickly transferred into liquid nitrogen (-196°C) and held for 15 min to 48 h. The samples were warmed either by direct transfer into a $+37^{\circ}\text{C}$ water bath ($\sim 500^{\circ}\text{C min}^{-1}$) or by transfer into stirred alcohol (300 ml in a 1-l beaker) initially at $\sim -110^{\circ}\text{C}$ and allowed to warm in air ($\sim 2^{\circ}\text{C min}^{-1}$). At different temperatures, samples were removed and warmed rapidly in a $+37^{\circ}\text{C}$ water bath until the ice disappeared. After thawing the samples were diluted rapidly⁵ and the embryos were washed in PB1 and cultured in Brinster's BMOC-3 medium⁶. Survival was measured as a percentage of cultured embryos developing to the expanded blastocyst stage in 48 h. Approximately 90% of the embryos frozen were recovered and cultured. Temperature was measured using copper-constantan thermocouples and a potentiometric recorder. Each data point represents five replicate tubes.



which intracellular ice was first observed to form. We also examined whether the functional injury was reversible, by repeating the experiments described in Fig. 2 legend, except that instead of warming the embryos rapidly from various temperatures during slow warming, they were recooled rapidly from those temperatures into liquid nitrogen and then rewarmed rapidly in a +37 °C water bath. The survival was not significantly different from that in Fig. 2. This suggests that the injury resulting from some event that occurs during slow warming could not be reversed by a return to a lower temperature before rapid warming.

These results indicate that the absence of intracellular freezing during cooling is no guarantee that freezing will not occur within the cells during warming. This finding has important implications for those using low-temperature techniques for preservation of either structure or function. The initial formation of intracellular ice at -85 °C during slow warming clearly did not result in cellular injury, as injury occurred only when slow warming was continued for a further 20 °C (to about -65 °C). Furthermore, the embryos were injured before the second darkening of the cytoplasm at -55 °C and the melting of the intracellular ice at -40 °C. If the three events of flashing at -85 °C, darkening at -55 °C and disappearance of the dark crystalline material in the cytoplasm at -40 °C correspond, respectively, to devitrification (crystallization)^{14,15}, migratory recrystallization¹⁵ and melting of ice in the cytoplasm, then our data showing injury at ~ -65 °C do not support a correlation of injury with these phase transitions of intracellular ice. This conclusion contradicts previous suggestions of the mechanism by which intracellular ice injures. Mazur has suggested that injury from intracellular ice and its migratory recrystallization is a direct physical consequence of the ice and the effect is on intracellular membranes^{8,16}. If this were so, the injury should occur at the same temperature as, or soon after, migratory recrystallization. Farrant has suggested instead that injury results from osmotic forces mediated by the imbalance between the melting of intra- and extracellular ice¹⁷; with this idea most injury should occur after the intracellular ice melts. Three alternatives may account for the discrepancy between our survival data and the phase changes of intracellular ice. First, an invisible event may occur at -65 °C (for example, recrystallization of ice) that leads to injury. Second, one of the visible changes of intracellular ice (recrystallization at -55 °C) may be the damaging event and become inevitable once slow warming reaches -65 °C. Finally, the presence of intracellular ice may be innocuous and functional injury result from a change in another component of the system.

We thank Dr C. R. Coid for providing research facilities and Miss C. Evans for the photographic processing.

Received 13 March; accepted 21 May 1980.

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Precursor complement protein (pro-C4) is converted *in vitro* to native C4 by plasmin

Gabriel Goldberger & Harvey R. Colten

Division Cell Biology, Department of Medicine, the Ina Sue Perlmutter Cystic Fibrosis Research Center, Children's Hospital Medical Center and the Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115

The fourth component of complement (C4) is a constituent of the classical serum complement pathway, one of the principle effectors of inflammatory reactions. A single chain protein (molecular weight (MW) 200,000), presumed to be the precursor of C4 (pro-C4), was first identified as a product of cell-free translation on polysomes from guinea pig liver homogenate¹. Pro-C4 has since been detected in human^{2,3} and guinea pig⁴ plasma and intracellularly in macrophage⁵ and liver cultures⁶. Native C4 is composed of three polypeptide chains of unequal size and linked by disulphide bridges—the α , β , and γ chains which have MWs of approximately 95,000, 75,000 and 31,000 respectively (ref. 6). Kinetic studies of C4 biosynthesis in tissue culture, a comparison of peptide maps from tryptic digests⁷ and identity of the amino-terminal segments of guinea pig pro-C4 with β chain of guinea pig and human C4 (ref. 8) suggested that pro-C4 was a precursor of C4. However, conversion of pro-C4 to native C4 has not been demonstrated directly. We now report that the addition of plasmin to radiolabelled pro-C4 (in cell lysates of guinea pig macrophages) generated a protein with the characteristic size and subunit structure of native C4, establishing the precursor-product relationship between these proteins. These data also suggest that a plasmin-like enzyme is responsible for post-translational conversion of this procomplement (pro-C4) protein to C4 *in vivo*. Another enzyme, the C1s subunit of the first component of complement, cleaved pro-C4 at a single site, distinct from the plasmin cleavage sites, to generate fragments of approximate MWs 112,000 and 91,000. This experiment indicated that β - α - γ is the sequence of subunits in pro-C4.

A previous study⁸, using a microradiosequencing method, showed that the β chain is the amino-terminal segment of pro-C4; the carboxy-terminal segment had not been ascertained. Hence, a preliminary study of pro-C4 structure was required to interpret results of experiments designed to convert the procomplement protein to native C4. Guinea pig macrophage cultures from starch-induced peritoneal exudates were incubated for 5 h in medium containing ³⁵S-methionine. The cell monolayers were then washed extensively and the intracellular radiolabelled protein containing pro-C4 was recovered in the supernatant following 100,000g centrifugation of a 0.5% Triton X-100 lysate of the cells. Culture medium served as the source of radiolabelled native C4.

The C1s subcomponent of the first component of complement is an enzyme that cleaves the amino terminus of α -chain of native C4 at a single site, liberating a fragment of MW 9,000 (C4a). To test whether this enzyme would be of use in deducing the sequence of subunits in the precursor protein, purified C1s was mixed in excess with radiolabelled pro-C4 in cell lysate or with ³⁵S-methionine-labelled native C4 secreted by macrophages in culture. After incubation for 60 min at 37 °C the products were subjected to SDS-polyacrylamide gel electrophoresis (Fig. 1). In confirmation of published data⁶, C1s treatment of native C4 resulted in alteration of the mobility of α chain (generation of α') due to loss of a 9,000-MW fragment. There was no apparent change in mobility of either β or γ chain. C1s also cleaved pro-C4 at a single site to generate two polypeptides of approximate MWs 112,000 (α' - γ) and 91,000 (β chain + 9,000-MW (9 K) fragment of α chain); no free α' chain

Fig. 1 Cleavage of pro-C4 and native C4 by C1s. Cell lysate (as a source of ^{35}S -methionine-labelled pro-C4) prepared as described in Fig. 3 legend and medium from the same cultures (as a source of radiolabelled native C4) were each mixed with purified C1s ($140\ \mu\text{g ml}^{-1}$ final concentration)¹² and the mixtures were incubated at 37°C for 60 min. Controls consisted of lysate or medium incubated in identical conditions except that C1s was omitted. C4 antigen was isolated by precipitation⁹ and the products of C1s action on pro-C4 and C4 were analysed by 1% SDS/9% polyacrylamide gel electrophoresis¹³. Lane 1, pro-C4; lane 2, pro-C4 treated with C1s; lane 3, native C4; lane 4, native C4 treated with C1s. The two fragments generated from pro-C4 by C1 cleavage were stable on prolonged incubation with the C1s enzyme. Their sizes (MWs 112,000 and 91,000) were estimated in a parallel experiment on cylindrical 1% SDS/5.6% polyacrylamide gels as described by Fairbanks *et al.*¹⁴. Δ , C1s cleavage site.

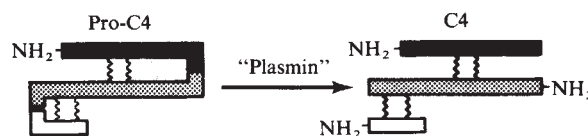
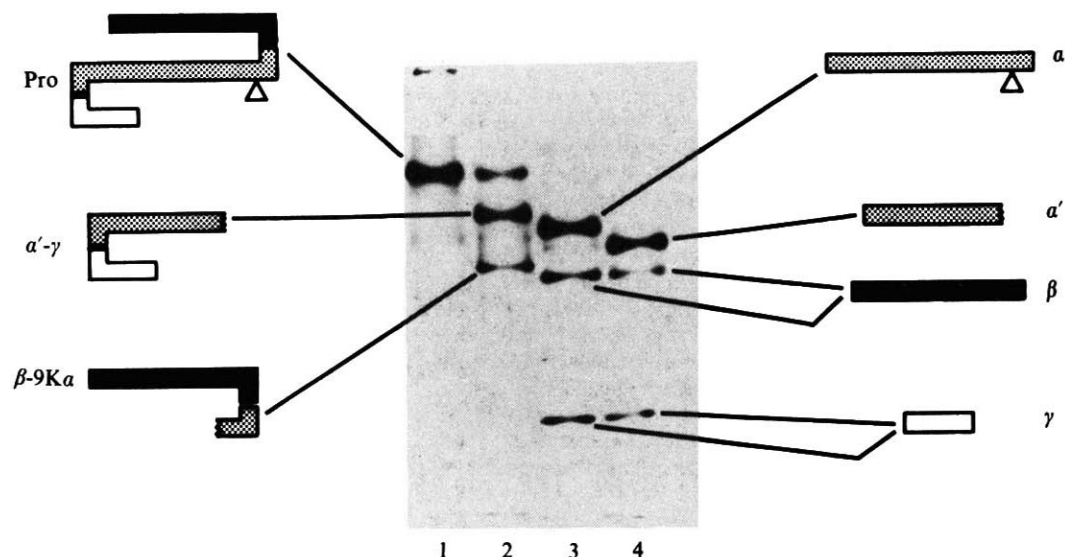
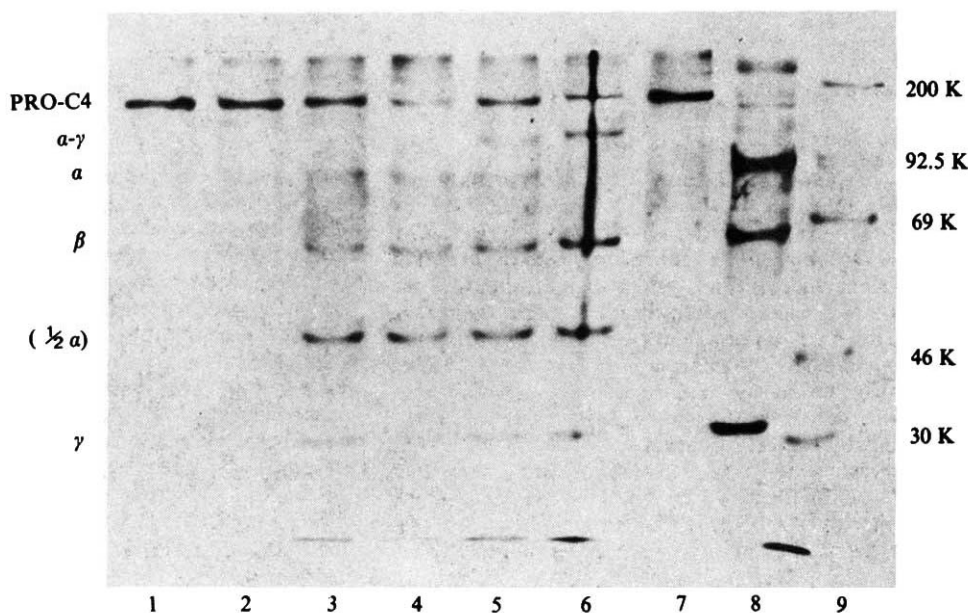


Fig. 2 Model of Pro-C4 with β chain (■) as the amino-terminal segment linked to α chain (□) followed by γ chain (□) at the carboxy terminus. Conversion of pro-C4 to C4 mediated by plasmin or a plasmin-like enzyme according to data in Fig. 3 legend. The position and number of disulphide bridges are not known.

Fig. 3 Kinetics of pro-C4 cleavage by plasmin. Confluent cultures of macrophages from starch-induced peritoneal exudates in Hartley guinea pigs were established by plating 3×10^7 cells in 100-mm plastic Petri dishes (Falcon Plastics) with 10 ml Medium 199 containing 10% heated fetal bovine serum (FBS), $50\ \text{U ml}^{-1}$ penicillin and $50\ \mu\text{g ml}^{-1}$ streptomycin (P/S). After 2 h incubation at 37°C in humidified 5% $\text{CO}_2/95\%$ air atmosphere, the monolayers were washed thrice with Hank's balanced salt solution (HBSS) then fed with 6 ml of supplemented (10% dialysed heated FBS, P/S and glutamine 2 mM) Dulbecco's modified Eagle's medium in which ^{35}S -methionine ($>500\ \text{Ci mmol}^{-1}$) $85\ \mu\text{Ci ml}^{-1}$ was substituted for the corresponding unlabelled amino acid. Culture medium was removed after 5 h and the cells were washed extensively with HBSS then lysed in 6 ml $\text{KC1 } 100\ \text{mM}$, $\text{Tris } 50\ \text{mM}$, $\text{pH } 7.5$ buffer with 0.5% Triton X-100. Following one freeze-thaw cycle, the lysate was centrifuged at $100,000\ \text{g}$ for 2 h at 4°C and the supernatant stored at -90°C . The cell lysate (1.7 ml) was mixed with $34\ \mu\text{l}$ plasmin stock solution prepared as described below. An aliquot ($300\ \mu\text{l}$) was immediately removed, mixed with benzamidine (20 mM final concentration) and stored at 0°C . The remainder was incubated at 37°C and at timed intervals $300\text{-}\mu\text{l}$ aliquots were removed, benzamidine added and the samples stored at 0°C . Controls consisted of lysate alone stored at 0°C , and lysate alone incubated at 37°C for 55 minutes. Sodium EDTA (2 mM final concentration) and a pseudoglobulin fraction of guinea pig serum⁹ were added to each sample, then the mixture was centrifuged $10,000\ \text{g}$ for 5 min at 4°C . C4 antigen was precipitated from $250\ \mu\text{l}$ of the supernatant according to the method in ref. 9. The precipitates were washed, dried and dissolved in $50\ \mu\text{l}$ of a solution containing 10 mM Tris, 1 mM Na_2EDTA , 1% SDS, 10% glycerol, $60\ \mu\text{g}$ pyronine-y, 4% 2-mercaptoethanol, boiled for 4 min then applied to a 1% SDS/9% polyacrylamide gel and electrophoresed¹⁴. The gel was stained, fixed and dried and an autoradiograph developed after 2 weeks at -70°C . Lane 1, lysate untreated; lanes 2-6, lysate treated with plasmin at 37°C for 0, 5, 10, 20, 55 min, respectively; lane 7, lysate incubated at 37°C for 55 min in the absence of plasmin; radiolabelled native C4 from medium (see Fig. 1 legend) in lane 8 and MW markers in lane 9. Note the disappearance of pro-C4 following plasmin treatment and appearance of α , β and γ chains of native C4. Note also the 130,000- and 50,000-MW fragments. Plasminogen (2 mg ml^{-1}) purified on lysine Sepharose¹⁵ was activated by incubation with urokinase (Abbot) 10% w/v for 30 min at room temperature. Plasmin activity was measured spectrophotometrically by hydrolysis of chromogenic substrate S-2251 (Ortho Diagnostics); $5\ \mu\text{l}$ of the stock solution liberated $4.7\ \text{mM s}^{-1}$. MWs ($\text{K} \times 10^3$) are given on the right-hand side.



was seen. These data are consistent with the model of pro-C4 shown in Fig. 2 with β chain, the amino-terminal segment, then α chain and γ chain at the carboxy-terminus.

Enzymatic conversion of pro-C4 to native C4 was accomplished in the following experiment. Plasmin (human plasminogen and plasminogen activator) was mixed with radiolabelled cell lysate and incubated at 37 °C. At timed intervals, aliquots were removed and the reaction stopped with benzamidine (20 mM final concentration). C4 antigen was precipitated⁹, and the precipitates were washed, dissolved and subjected to SDS-polyacrylamide gel electrophoresis in reducing conditions (Fig. 3). Treatment with plasmin resulted in a loss of pro-C4 protein and the appearance of disulphide-linked peptides with electrophoretic mobilities identical to the mobilities of the α , β and γ chains of unlabelled carrier C4 protein, mixed and co-electrophoresed with the samples. In addition, a protein of approximately 50,000 MW, interpreted to be a fragment of α chain (1/2 α), appeared. This interpretation was supported by the observation that the intensity of the α -band decreased, whereas β and γ chains were stable during plasmin treatment. An additional protein fragment consistent in size

(MW 130,000) with an α - γ segment was also generated. The rate of cleavage of pro-C4 by plasmin was a function of enzyme concentration and temperature, and was inhibited by benzamidine (20 mM). Cleavage of pro-C4 was not observed in cell lysates alone, that is, those lacking plasmin, nor in the presence of plasminogen activator (human urokinase) alone. Moreover, the C1r subcomponent of C1, properdin factor D and urinary kallikrein each failed to generate C4 protein from pro-C4. Trypsin extensively degraded pro-C4 to a mixture of small molecular weight peptides.

The possibility that a plasmin-like enzyme converts pro-C4 to C4 *in vivo* is of interest in view of evidence that levels of plasminogen activator in monocytes¹⁰ and macrophages¹¹ are modulated by a variety of stimuli, thus providing a potential mechanism for post-translational control of complement production.

We thank Drs Brian Tack for supplying plasminogen, Judy Andrews for purified C1s and C1r, Jocelyn Spragg for purified human urinary kallikrein, and A. E. Davis III for purified factor D. This work was supported by USPHS grants AI15033 and HL22487.

Received 24 March; accepted 28 May 1980.

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A specific enzyme is not necessary for vanadate-induced oxidation of NADH

F. Vyskočil*, J. Teisinger† & Hana Dlouhá*

* Institute of Physiology, Czechoslovak Academy of Sciences, 142 20 Prague 4-Krč, Videnska 1083, Czechoslovakia

† Institute of Hygiene and Epidemiology, Prague 2, Czechoslovakia

It has recently been found that ortho- or metavanadate can effectively block $(\text{Na}^+ + \text{K}^+)\text{ATPase}^{1-4}$ and that it loses its blocking potency when reduced to the vanadyl (VO^{2+}) ion⁵. The question arose whether vanadate could be involved (reduced) in an NAD-linked enzymatic redox system of the cell. Here we have studied the effect of vanadate on malate dehydrogenase (MDH, EC1.1.1.37) catalysed oxidation of NADH during the formation of malate from oxalacetate *in vitro*. The MDH reaction was accelerated by vanadate, but we found that vanadate does not require the presence of any specific enzyme or substrate to mediate NADH oxidation.

Unless otherwise stated, the reaction medium contained 1×10^{-4} mol l^{-1} NADH and 8×10^{-4} mol l^{-1} Na_3VO_4 [stock solution: 500 mg of NaVO_3 (Merck) dissolved in 1 ml of 1 mol l^{-1} NaOH] at the beginning of each experiment.

The rate of the MDH reaction (MDH 0.033 U ml^{-1}), measured continuously on an Eppendorf photometer at A_{366} as the amount of NADH lost in 0.1 mol l^{-1} Tris-HCl buffer (pH 7.2) at 37 °C, was accelerated in the presence of vanadate. The loss of NADH was, however, observed even without MDH, if only vanadate and NADH (and oxalacetate 0.5 mmol l^{-1}) were present in the reaction medium. The vanadate-accelerated MDH reaction (loss of 4.14 μmol of NADH per min) is thus the result of the sum of the MDH reaction (loss of 1.75 μmol of NADH per min) plus the vanadate-NADH (loss of 2.29 μmol of NADH per min). Vanadate can thus induce the oxidation of NADH (probably by accepting electrons itself), and this process does not require any further enzymatic support. According to

our preliminary observations in the UV spectrophotometer, vanadate immediately forms a complex with NADH, within which the NADH reduction subsequently proceeds. This redox system might be masked in biochemical experiments in which buffers with EDTA (or biological samples containing this

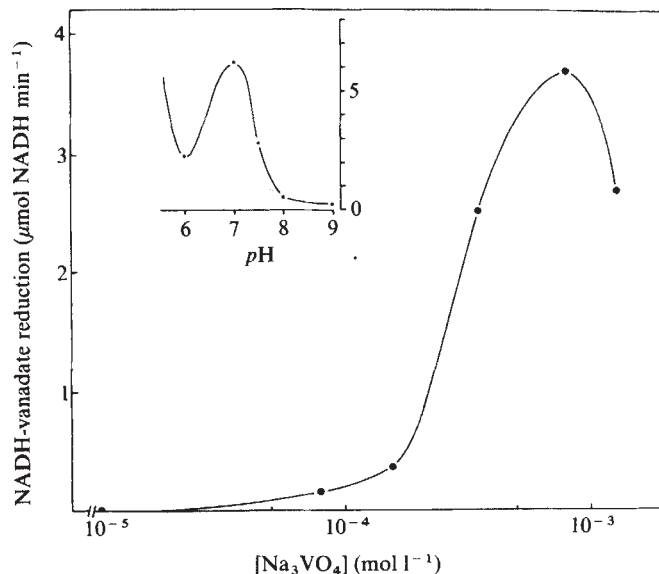


Fig. 1 Effect of vanadate on NADH oxidation in 0.1 mol l^{-1} phosphate buffer (pH 7.3) at 37 °C. Abscissa, concentration of vanadate $[\text{Na}_3\text{VO}_4]$ in mol l^{-1} . Ordinate, the rate of NADH-vanadate reduction expressed as loss of NADH in $\mu\text{mol min}^{-1}$ (see Erdmann *et al.*⁷, Fig. 1). Inset, pH dependence of NADH loss (in $\mu\text{mol NADH per min}$, ordinate) in 0.1 mol l^{-1} phosphate buffer at 37 °C. Concentrations of vanadate and NADH at the beginning of experiments were 8×10^{-4} and 1×10^{-4} mol l^{-1} , respectively. All results presented here are the means of 5-10 experiments. The s.e.m. values were always smaller than 5% of the mean and are therefore not indicated.

Table 1 The rate of NADH oxidation in the presence of 8×10^{-4} mol l⁻¹ vanadate in different reaction media

	mol l ⁻¹	T(°C)	pH	-ΔNADH
Na-phosphate	0.1	17	7.3	2.18
Na-phosphate	0.1	27	7.3	2.41
Na-phosphate	0.1	37	7.3	3.84
Na-phosphate	0.1	37	7.0	6.15
Tris-HCl	0.1	37	7.2	5.20
HEPES-NaOH	0.01	37	7.0	1.70
Liley's solution	*	37	7.3	0.92
Histidine-NaOH	0.01	37	7.1	0.09
Imidazole-HCl	0.01	37	7.0	0.00

The rate of NADH oxidation is expressed as the loss of NADH in $\mu\text{mol min}^{-1}$, $-\Delta\text{NADH}$. The concentration of NADH at the beginning of experiments was 10^{-4} mol l⁻¹.

The composition of Liley's solution⁹ was (mol l⁻¹): Na⁺ 149.8; K⁺ 5.0; Ca²⁺ 2.0; Mg²⁺ 1.0; Cl⁻ 148.0; HCO₃⁻ 12.0; H₂PO₄⁻ 1.0; glucose 11.0.

chelating compound) are used; in the presence of 1×10^{-3} mol l⁻¹ EDTA no oxidation of NADH was observed, probably because inactive complexes are formed with vanadate⁶.

A recent report⁷ described the vanadate-NADH reductase activity of cardiac cell membranes. Because these authors claimed that there was no vanadate-induced NADH oxidation unless membrane proteins were present, we looked for the probable sources of this discrepancy. The only difference between our experiments and those of Erdmann *et al.*⁷ was the buffer used in the reaction medium. When we started the vanadate-NADH reaction in a 0.01 mol l⁻¹ imidazole-HCl buffer⁷, we also found no oxidation of NADH. Contrary to the findings of Erdmann *et al.*⁷, we observed no vanadate-dependent oxidation of NADH in the presence of membrane fractions isolated from rat and golden hamster heart⁸ and other tissues (skeletal muscle, brain and liver). Subsequently, we checked several other buffers used for biochemical experiments (Table 1) and Liley's extracellular medium⁹. With the exception of the imidazole and histidine buffer, the vanadate-NADH reaction proceeded in all media.

The vanadate-NADH reaction in Tris-HCl medium was slowed to 50% by either 2.5 mmol l⁻¹ Ca²⁺ or 20 mmol l⁻¹ Mg²⁺. The presence of Ca²⁺ and Mg²⁺ might therefore explain the rather slow rate of NADH oxidation in Liley's medium. The reaction is dependent on the concentration, pH (Fig. 1) and temperature (Table 1).

Similar results were obtained in the NADPH-vanadate reaction medium. Thus, 2.95 μmol NADPH was oxidized within 1 min in the presence of 8×10^{-4} mol l⁻¹ vanadate and 7×10^{-5} mol l⁻¹ NADPH at 37 °C in the Tris-HCl buffer.

Further examples can be presented concerning the more generalized role of vanadate in living redox systems. Vanadate-NADPH reduction was accelerated by 80% after the addition of 1 $\mu\text{mol l}^{-1}$ β -hydroxy- β -methyl-glutaryl-CoA (HMGCoA). However, it is not clear whether the reduction of HMGCoA to mevalonate is triggered in this particular case by vanadate in the absence of hydroxymethylglutaryl CoA reductase or whether HMGCoA only accelerates the transfer of electrons from NADH to vanadate within the complex. Whatever the explanation, this effect of vanadate might be of particular interest because the HMGCoA reductase is generally considered to be the rate determining enzyme for the conversion of acetate to cholesterol¹⁰.

Received 31 January; accepted 27 May 1980.

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Visualization of the structural polarity of microtubules

Steven R. Heidemann* & J. Richard McIntosh†

* Department of Physiology, Michigan State University, East Lansing, Michigan 48824

† Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80309

Microtubules (MTs) are intrinsically polar fibres¹⁻⁸. Many models for the mechanism of MT-mediated transport and mitosis postulate an important role for MT polarity^{7,9-12} because a symmetric fibre could not generate force in one direction along its surface. The lack of a convenient method to determine the polar orientation of cellular MTs has hindered experimental testing of these ideas. We report here a convenient technique for visualizing MT polarity based on conditions for tubulin assembly *in vitro* whereby MT elongation occurs with the associated formation of hooked, protofilament appendages on the MT wall (see Fig. 1). There is polarity information in such junctions between MT walls¹³⁻¹⁵; our method reveals the polarity of the cellular MT by the handedness of the hooked appendage as seen in cross-section (Fig. 1).

Microtubule protein (MTP) was prepared from porcine brain by the method of Shelanski *et al.*¹⁶. After three cycles of polymerization, the protein was resuspended in 0.5 M PIPES pH 6.9, 2.5% dimethyl sulphoxide, 1 mM EDTA, 1 mM MgCl₂, and 1 mM GTP (hereafter called polymerization buffer (PB)) at 0 °C and spun at 250,000g for 3 h. The polymerization of MTP in PB is similar in its magnesium and GTP requirement and in its cold, calcium and colchicine lability to that which occurs in other polymerization buffers¹⁷. MTP will assemble to the same extent in PB in the presence of a strong detergent mixture, 1% Triton X-165, 0.5% deoxycholate, 0.2% SDS. MTP assembly in PB also shows the biased polar growth characteristic of other *in vitro* assembly conditions^{5,7,18}. One end of isolated *Tetrahymena* ciliary axonemes elongated about twice as fast as the other at the protein concentrations tested. Elongation at each end of the axoneme was about three times as fast as growth at the comparable end in 0.1 M PIPES buffer.

The polymer initiated *de novo* in PB consists primarily of ribbons of laterally associated protofilaments, as previously reported for similar conditions^{14,15}, not true MTs. However, when true MTs nucleate the polymerization, the elongated polymer usually has an evident lumen, and ribbons of protofilaments join the MT wall, forming hooked appendages (Fig. 1). We have examined such polymers to see if one handedness of hook predominates by nucleating MT growth from basal bodies. The MTs of the basal body are known to be oriented with the fast growing end of their MTs pointing away from the cell⁶. When lysed and deciliated *Tetrahymena* were used to nucleate the assembly of MTP in PB, polymerization occurred largely as elongation from the basal bodies (Fig. 2a). Pellicles in MTP fixed before polymerization was initiated showed no MT growth (Fig. 2b). After polymerization, the organized geometry of basal bodies in the pellicle allowed us to observe the elongated polymers in known orientations with respect to the basal bodies that initiated them (Fig. 2c). We found that about 95% of the newly grown MTs had the hook-shaped appendages. Figure 2d is a composite of several such images as seen looking toward the basal body from outside the pellicle. Figure 2e shows MTs growing inward as seen from the basal body. We confirmed both in longitudinal sections and by negative staining that the hooks are protofilament sheets which make a junction with the MT wall. The cross-section images demonstrate that when the elongated MT is viewed from its fast growing end or '+end' towards its slow growing end or '-end'(ref. 8) the hooks are commonly right handed. On polymers growing outward from the basal body (those initiated at a fast end) we counted 562 MTs with right-handed hooks and 62 with left-handed hooks when viewed from +end to -end. On

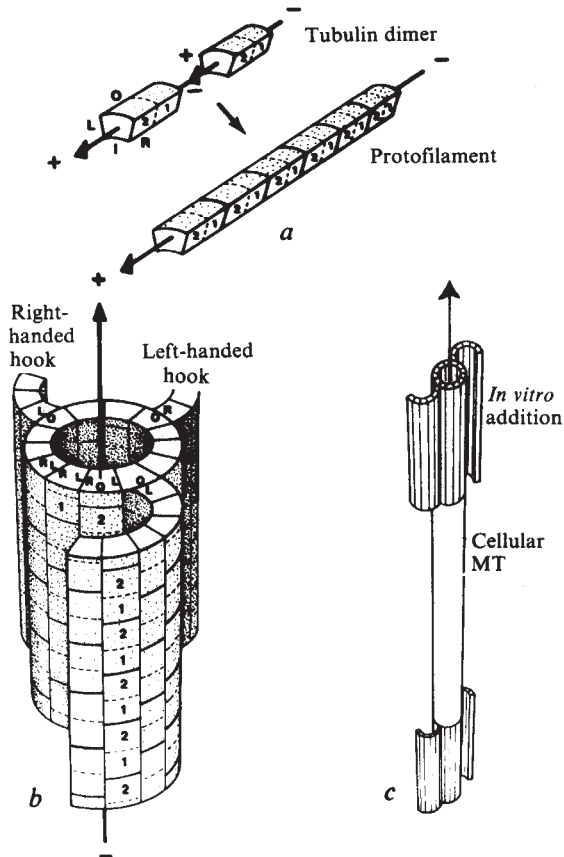


Fig. 1 Different associations of MTP. The tubulin dimer is pictured as having six dissimilar surfaces. + and - correspond to the distal and proximal ends, respectively, of a flagellar MT²². I and O are the surfaces which would be on the inside and outside of a normal MT, L and R are the two surfaces which associate laterally in the wall of a normal MT. The numbers 1 and 2 denote the different subunits of the tubulin dimer. The intradimer bonds are suggested by dashed line, interdimer bonds by solid ones. *a*, The head-to-tail association of dimers to form protofilaments. *b*, Protofilaments have associated laterally to form MT wall (L-R interactions) or in two alternative geometries to form junctions between MT walls, which also occur naturally^{23,24}. Wall junctions and concomitant hooks of two possible hands are made by associating either the R or L surface of a protofilament with the O surface. A tacit assumption is made that the protofilament polarity of the hook wall is the same as that of the tubule itself. *c*, In our conditions neurotubulin adds *in vitro* to a cellular MT to form predominantly right-handed hooked MTs. The polarity orientation of a MT end can be determined from the observed hook-handedness of newly grown MTs.



Fig. 2 *Tetrahymena* pellicles were used to nucleate growth of 1 mg ml^{-1} microtubule protein in PB at 37°C for 6 min, then fixed by addition of an equal volume of 2% glutaraldehyde in 0.1 M PIPES. Fixed pellicles were allowed to settle onto polylysine-coated slides and were then treated with 0.2% tannic acid, rinsed in phosphate-buffered saline, post-fixed in $\frac{1}{2}\%$ OsO_4 for 2 min, dehydrated in 2-methoxyethanol and flat embedded in Epon. Individual pellicles were excised, serially sectioned and stained with uranyl acetate and lead citrate. Microscopy was performed on a Phillips 300 equipped with a goniometer stage to facilitate orientation of the tubules. *a* Is a Nomarski image of MT polymerization from such a pellicle after 6 min at 37°C , $\times 600$. *b* Was fixed at 0°C (no growth), $\times 55,000$. *c* Is 6 min of growth. The orientation of the growing MTs may be deduced from pellicle geometry, $\times 55,000$. *d* Is a composite of MT images seen looking from the outside of the pellicle towards the basal body, $\times 92,000$. *e* Shows MTs growing inward from the basal body; *d* and *e*, $\times 92,000$.

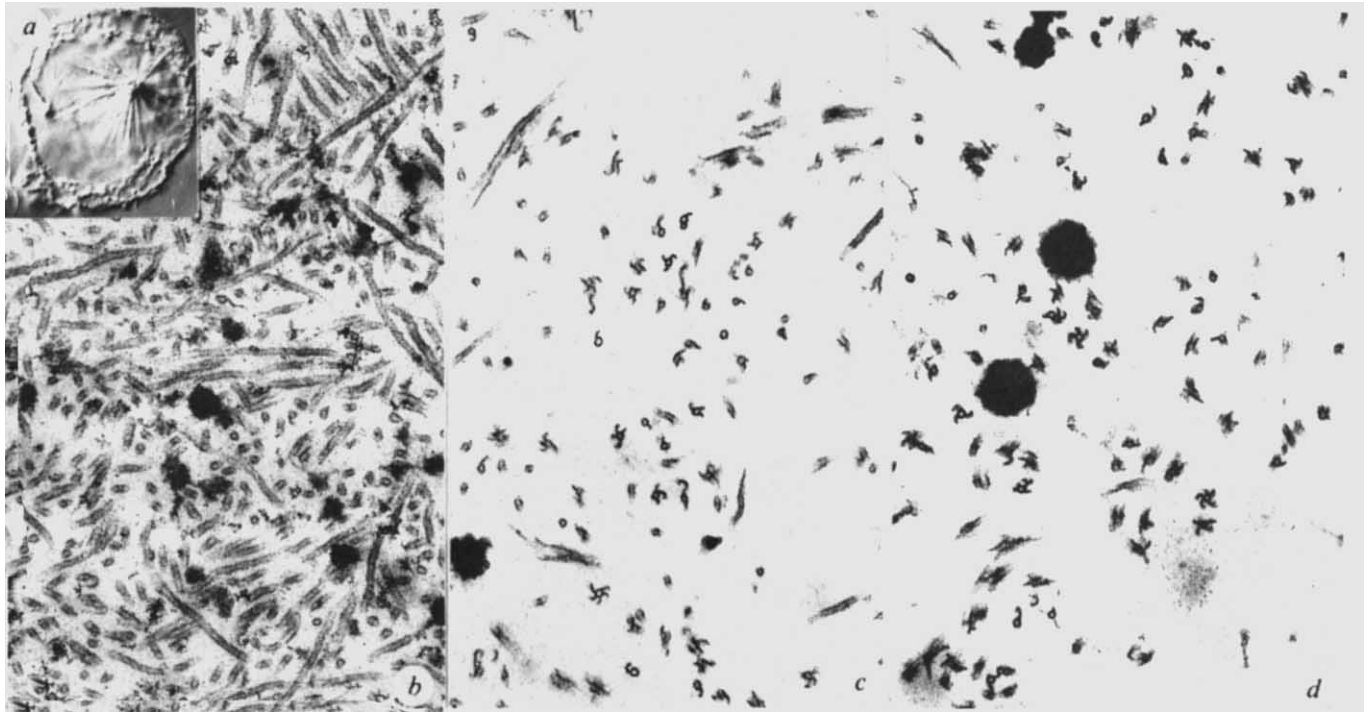


Fig. 3 Microtubule polarity in the mitotic aster of the HeLa cell. *a*, Nomarski image of a metaphase-arrested HeLa cell lysed in detergent-containing PB with 2 mg ml^{-1} MTP, $\times 750$. *b*, An electron micrograph from a section approximately at the centre of an aster. Note that MTs are generally of normal morphology. *c*, The periphery of an aster in a section 'before' the aster centre. Note that most MTs have right-handed hook appendages. *d*, The periphery of an aster in a section 'beyond' the aster centre. The hooks are now left handed; however, the apparent observation point for these hooks is now proximal to the centre, in contrast to the distal vantage point of *c*; *b*, *c* and *d*, $\times 40,000$.

MTs growing inward from the basal bodies, viewed from the same direction, we found 135 MTs with right-handed hooks and 25 MTs with left-handed hooks. Eight MTs displayed hooks of both handedness.

We next studied the polarity of mitotic MTs by using a remnant of the mitotic apparatus from HeLa cells stably blocked in metaphase with $0.04 \mu\text{g ml}^{-1}$ of the microtubule poison nocodazole¹⁹ contained large numbers of MTs with typical mitotic organization²⁰. Lysis of nocodazole-poisoned cells in PB containing the detergent mixture described above preserved metaphase aster structures approximately $5 \mu\text{m}$ in diameter surrounded by a thin, fibrous cell remnant. These asters consisted of normal MTs, presumably those which existed in the drug-poisoned cell. Inclusion of 2 mg ml^{-1} MTP in the PB lysis buffer produced asters up to $20 \mu\text{m}$ in diameter (Fig. 3*a*). In these large asters, 85% of the MTs were normal within a $5 \mu\text{m}$ radius of the aster centre (Fig. 3*b*). In the area beyond a $5 \mu\text{m}$ radius, 80% of the MTs displayed the hooked appendages (Fig. 3*c,d*). Our interpretation is that exogenous brain tubulin added to the distal ends of pre-existing HeLa MTs to form hooked MTs. Serial sections from four such asters reveal predominately right-handed hooks when viewed looking toward the aster centre: 1752 MTs showed only right-handed hooks, 121 MTs bore only left-handed hooks and 66 MTs displayed hooks of both handedness.

Close to 90% of all hooks on neurotubules elongated from both basal body and astral MTs displayed a consistent handedness relative to the MT on which they formed. As the same predominant handedness of hook was seen on MTs growing in both directions from the basal body, we conclude that our method reveals the molecular polarity of a MT, not its direction of growth. For the same reason, we infer that the MTs which grew from the basal body have the same polarity as the A subfibre in the basal body itself. Hook handedness on the elongated MT therefore reflects the polarity of the MT which nucleated elongation. The preponderance of right-handed hooks on MTs extending from both asters and basal bodies indicates that the astral MTs have the same polarity relative to the mitotic centre as do the outer nine flagellar MTs relative to

the basal body. That is, the fast or +end of the HeLa MT is distal to the mitotic centre. This is the same polarity as that of neurotubules initiated from the mitotic centres of lysed CHO cells^{21,22}. This agreement supports our assertion that hook handedness is a valid marker of MT polarity. The observed bias toward right-handed hooks on MTs grown from both basal bodies and asters was strong (about 9:1) but not complete. Further, MTs with hooks of both hands were occasionally observed, so we conclude that left-handed wall junctions are not excluded. Our method reveals and uses a biased formation of right-handed wall junctions to visualize the molecular polarity of the MT.

This investigation was supported by Biomedical Research Support funds from the Colleges of Osteopathic Medicine and Natural Science, Michigan State University, and grants CD 8D from the American Cancer Society and PCM-77-14796 from the NSF to J.R.M. S.R.H. would like to thank the Department of Microbiology, M.S.U. for the use of their electron microscope.

Received 17 March; accepted 13 May 1980.

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N-terminal amino acid sequence homology of storage protein components from barley and a diploid wheat

Peter R. Shewry*, Jean-Claude Autran†, Charles C. Nimmo, Ellen J.-L. Lew & Donald D. Kasarda

Food Proteins Research Unit, Western Regional Research Center, Agricultural Research, SEA, US Department of Agriculture, Berkeley, California 94710

Wild barley (*Hordeum spontaneum*) and the wild diploid wheat *Triticum boeoticum* were possibly the first plants cultivated by early man¹, giving rise to the domesticated forms *Hordeum vulgare* L. and *Triticum monococcum* L. In addition, *T. boeoticum* may have contributed the A genome to polyploid wheats, including common bread wheat (*Triticum aestivum*)² which is a hexaploid with genome composition ABD. *Hordeum* seems to be the older genus, having diverged from some common ancestor before the divergence of *Triticum* and other genera of the subtribe Triticinae³. Prolamins constitute the major storage protein fraction of both barley and wheat; they are located in the endosperm of the caryopsis and are soluble in alcohol-water solutions⁴. Barley and wheat prolamins (hordeins and gliadins, respectively) contain large amounts of glutamine and proline, which together make up 50–75 mol per cent of total amino acids^{4,5}. The hordeins and gliadins are complex mixtures of components^{6–8} that seem to be encoded by clusters of duplicated genes that have diverged to produce many distinguishable protein components. Despite the complexity of the gliadin mixture, the components retain considerable homology in their N-terminal region^{9,10} and this has been reported for zeins, the prolamins of maize (*Zea mays*)¹¹, as well. Here, we report that a purified C-hordein component from barley is homologous in amino acid sequence with a purified ω -gliadin component from *T. monococcum* at 23 of 27 residues at the N-terminus. This result is in accord with the close relationship between the two species and indicates that, despite the propensity of prolamin genes to tolerate mutations, a significant portion of their sequences can be conserved over a period of time, which, although not accurately known, probably amounts to millions of years.

Prolamins were extracted from barley (*H. vulgare* Julia) and *T. monococcum* (from the University of Manitoba) as described previously^{10,12}. The C-hordein component was obtained by ion exchange chromatography of the hordein mixture on CM-cellulose followed by gel filtration on Sephacryl S-300 (ref. 12). The ω -gliadin component from *T. monococcum* was obtained by ion exchange chromatography of the gliadin mixture on CM-cellulose according to the procedure of Booth and Ewart¹³ except that the gradient ranged linearly from 5 to 43 mM in sodium acetate; this component was not purified further. Electrophoretic patterns of the purified components in aluminium lactate buffer, pH 3.2, where separation is based on net charge, are compared with those of the prolamin mixtures in Fig. 1. We estimated molecular weights of 57,000 for the C-hordein and 44,000 for the ω -gliadin by SDS polyacrylamide gel electrophoresis⁶.

Amino acid analyses were carried out on a Durrum analyser, model D-500. Hydrolyses were for 24 h, tryptophan was not

determined, and no corrections for destruction of labile amino acids were applied. Averaged results from duplicate analyses are given in Table 1. Both components were notable for their high glutamine and proline content (although glutamic acid only is shown in Table 1, this amino acid is present in C-hordein¹² and probably in the ω -gliadin¹⁴ almost entirely in its amidated form^{12,14}). The C-hordein had more proline and less glutamine than the ω -gliadin, but the sum of these two amino acids was close to 70 mol per cent for both components. Both components had about 9 mol per cent phenylalanine, only trace amounts of cystine/2, and lysine was absent from the C-hordein and present in the ω -gliadin in an amount corresponding to only about 0.5 residue on a molar basis. Low percentages of basic amino acids, combined with equivalent low percentages of free carboxyl side chains^{12,14}, indicate that these proteins will have few charged side chains at any pH.

Automatic amino acid sequencing¹⁵ was carried out with a Beckman sequencer, model 890B, and DMAA peptide program 111374 (ω -gliadin) or 0.1 M Quadrol programs 011576 and 121178 (C-hordein). Duplicate analyses were carried out for each protein. In the first analysis of the ω -gliadin, identification of the phenylthiohydantoin (PTH) amino acids resulting from the Edman degradation¹⁵ was by gas chromatography¹⁶ only; this method was supplemented in the second analysis by TLC on polyamide sheets¹⁷ and silica gel plates¹⁸ and by hydrolysis to the free amino acid followed by analysis on the Durrum analyser. PTH amino acids from sequencing of the C-hordein were identified by HPLC¹⁹, which clearly resolved and quantified all the PTH amino acids found in the first 28 cycles. The N-terminal sequences obtained are compared in Fig. 2. Initial yields in sequencing ranged from 50 to 76% of molar

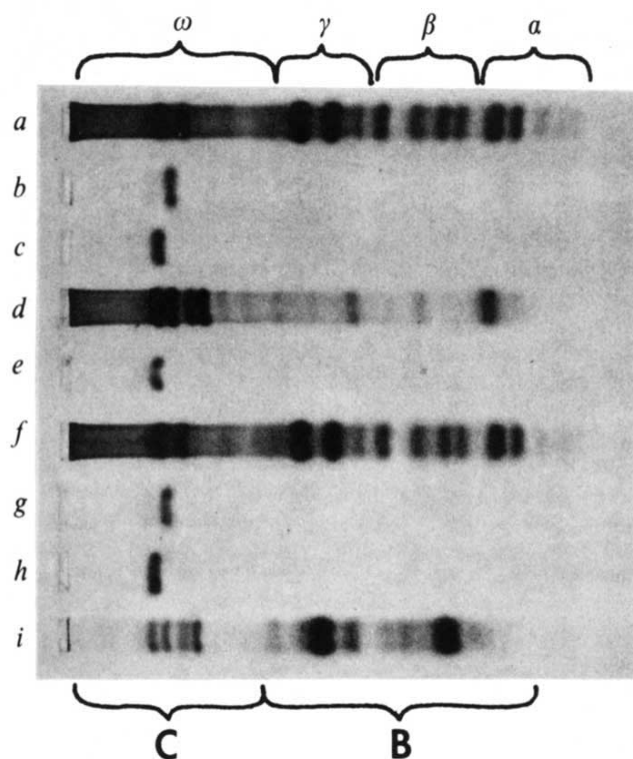


Fig. 1 Polyacrylamide gel electrophoresis²⁷ of purified protein components and the whole prolamin mixtures from which they were prepared, aluminium lactate buffer, pH 3.2, 3 M urea, migration from left (+) to right (-): a, prolamin mixture from *T. monococcum*; b, purified ω -gliadin from *T. monococcum*; c, purified C-hordein; d, prolamin mixture from barley (var. Julia); e, same as c; f, same as a; g, same as b; h, same as c; i, prolamin mixture from barley (Julia), but treated with 2% 2-mercaptoethanol to dissociate B-hordeins. The α -, β -, γ - and ω -regions (top) correspond to the usual assignments of electrophoretic mobilities for gliadin patterns of common wheats³ and the B- and C-regions (bottom) correspond to the hordein patterns.

* Permanent address: Biochemistry Department, Rothamsted Experimental Station, Harpenden, Herts AL5 2JQ, UK.

† Permanent address: Institut National de la Recherche Agronomique, Laboratoire de Technologie des Cereales, 9 Place Viala, 34060 Montpellier, France.

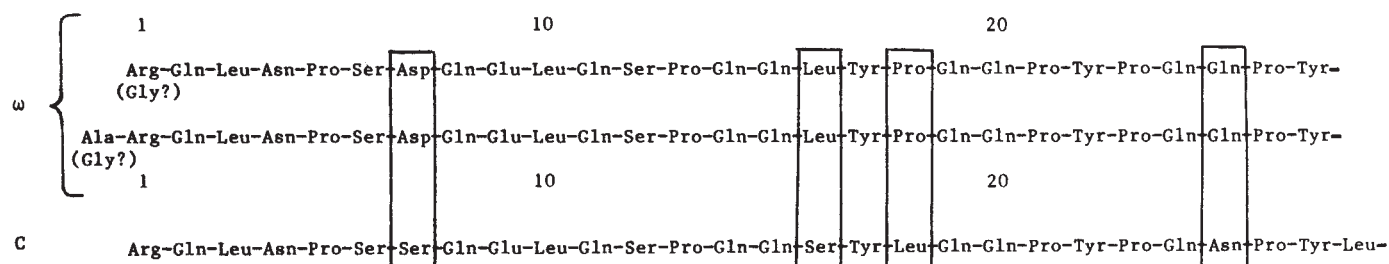


Fig. 2 N-terminal amino acid sequences of the ω -gliadin component from *T. monococcum* (ω) and the C-hordein component from barley (C). Sequences are ordered from the first arginine residue to emphasize homology. Glycine was a minor residue at cycle 1 in the sequence of the ω -gliadin. Positions where the ω -gliadin and C-hordein sequences differ are marked.

amounts applied. The highest yield of 76% for one of the C-hordein analyses indicates there was no significant N-terminal blocking.

No important minor residues were noted in sequencing the C-hordein, but three amino acids were identified in the first cycle of the ω -gliadin sequence and two amino acids at each subsequent cycle. Alanine was the major residue at cycle 1 in the ω -gliadin, but glycine and arginine were clearly identified; in subsequent cycles it became clear that the second sequence appearing at about half the level of the major sequence was the same as that of the major sequence displaced by one cycle. It evidently resulted from a protein component without the terminal alanine (or glycine) residue. Electrophoresis of the purified ω -gliadin did not show any minor components in sufficient amount to account for the second sequence; however, it is unlikely that a component differing by only one neutral amino acid would be resolved. The extra residue could mean that at least two genes code for the proteins in our preparation, but post-translational modifications might modify a single component in such a way as to produce the 2:1 ratio of components we observed.

Comparison of the amino acid sequences of our two components (Fig. 2) shows that they differ only at four positions if we do not consider the N-terminal alanine residue that is present in part of the ω -gliadin preparation. This demonstrates a close relationship between *H. vulgare* and *T. monococcum*. No fossil evidence provides a measure of the time since divergence of *Hordeum* and *Triticum*. If we assume for purposes of speculation, however, that the rate of evolution of the N-terminal regions of our proteins is equivalent to that of one of the fastest evolving peptide systems characterized so far, the fibrinopeptides (9.0 amino acid substitutions per site per 10^9 yr)²⁰, then the time since divergence of our two species would be 16 Myr.

The similar sequences we obtained for our C-hordein and our ω -gliadin from *T. monococcum* show little homology with any other N-terminal sequences reported for α -, β - or γ -gliadins of common wheat^{9,10,21,22}, rye prolamins¹⁰ or maize prolamins¹¹. Our sequence is the first reported, however, for any ω -gliadin component. Autran *et al.*¹⁰ did not note evidence of our ω -gliadin sequence in sequencing of the whole prolamins mixture from the same accession of *T. monococcum*, but this is not surprising as ω -gliadins probably constitute less than 10% of the total mixture. Our C-hordein sequence is closely similar to sequences recently obtained for the total hordein mixture²³, the C-hordein mixture²⁴ and a partially purified C-hordein component²⁵.

In common bread wheat, which is hexaploid (genomes A, B and D), gliadin proteins are encoded by genes located on homoeologous (partially homologous, but non-pairing in the polyploid) chromosomes 1 and 6 of each genome^{7,26-28}. Shepherd²⁹ has suggested that all gliadin genes were located originally on one chromosome and that translocation of part of this chromosome to another gave rise to the common ancestor that, in turn, differentiated into the progenitors of the common wheat genomes A, B and D. This common ancestor must have already differentiated from the line that gave rise to *Hordeum* as all the prolamins genes of barley are located on chromosome 5 with the B-hordeins and C-hordeins being coded by two separate, but linked, loci³⁰. Our results suggest that the C-hordein locus of barley and the ω -gliadin loci located on homoeologous chromosomes of group 1 in common wheat are homologous. It is also possible that the B-hordein locus is homologous with the gliadin loci coding for α -, β - and γ -gliadins that are located on homoeologous chromosomes of group 6 in common wheat but this is more difficult to establish as B-hordein is blocked at the N-terminus^{12,25}.

We thank A. Noma for amino acid analyses, B. J. Mifflin for barley samples, and B. L. Jones for the sample of *T. monococcum*. P. R. Shewry and J.-C. Autran are visiting scientists in receipt of NATO postdoctoral research fellowships. Reference to a company or product name by the Department is only for information and does not imply approval or recommendation to the exclusion of others that may also be suitable.

Received 2 April; accepted 21 May 1980.

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Table 1 Amino acid compositions (mol%) of the ω -gliadin protein from *T. monococcum* and the C-hordein protein from barley (var. Julia)

	ω -Gliadin	C-hordein
Asp	1.18	0.83
Thr	1.08	0.99
Ser	4.89	2.61
Glu	45.2	41.1
Pro	26.2	31.9
Gly	0.89	0.41
Ala	1.57	0.69
Val	0.80	1.11
Met	0.13	0.22
Ile	2.22	3.02
Leu	4.70	4.31
Tyr	1.17	2.29
Phe	8.09	9.03
His	0.56	0.60
Lys	0.13	0.0
Arg	1.04	0.84
Cys/2	Trace	Trace

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Structure of *S. platensis* [2Fe-2S] ferredoxin and evolution of chloroplast-type ferredoxins

Keiichi Fukuyama*, Toshiharu Hase†, Satoshi Matsumoto*, Tomitake Tsukihara*, Yukiteru Katsube*, Nobuo Tanaka‡, Masao Kakudo‡, Keishiro Wada† & Hiroshi Matsubara†

* Faculty of Engineering, Tottori University, Tottori 680, Japan

† Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka 560, Japan

‡ Institute for Protein Research, Osaka University, Suita, Osaka 565, Japan

Ferredoxins are iron-sulphur proteins with either one or two [4Fe-4S] clusters or one [2Fe-2S] cluster and they function as electron carriers in diverse metabolic systems. Extensive comparative studies on the primary structures of ferredoxins with low oxidation-reduction potentials from a wide variety of organisms have provided information on the structure-function and evolutionary relationships of these proteins^{1,2}. In addition, the three-dimensional structure of *Peptococcus aerogenes* 2[4Fe-4S] ferredoxin has been elucidated³. However, nothing is known about the three-dimensional structure of [2Fe-2S] ferredoxins such as those of chloroplasts. Here we report the three-dimensional structure of a [2Fe-2S] ferredoxin of *Spirulina platensis*, a blue-green alga, determined by X-ray crystallography at 2.5 Å resolution, and discuss the characteristic features of the molecule in relation to the molecular evolution of ferredoxins of this class.

We will give only a brief description of our study, because full details of the analytical procedures and crystal structure of ferredoxin will be reported elsewhere. The crystals were

orthorhombic with a space group C222₁ of $a = 62.32$, $b = 28.51$, $c = 108.08$ Å and $Z = 8$. Intensity data, including both reflections of each Friedel pair, were collected for native and uranium derivative crystals on a Rigaku four-circle diffractometer with Ni-filtered CuK α radiation by an ω scan procedure. After correction for absorption⁴ and radiation damage, the intensities of equivalent reflections were averaged. The heavy-atom parameters⁵ were refined by the F_{HLE} method⁶ with empirically determined values of the real/imaginary scattering ratio to give an R value ($R = \sum |F_{HLE} - f_H| / \sum |F_{HLE}|$) of 0.398 for 1,956 reflections (55% of total reflections). The best phase angle was calculated by the single isomorphous replacement method⁷ coupled with the anomalous dispersion method⁸, where the anomalous dispersion effect of the two iron atoms was taken into account. The mean figure of merit at 2.5 Å resolution was 0.58 for 2,182 reflections (61% of total reflections). A Kendrew-type skeletal model was built up using a Richards box⁹ on the basis of the best electron density map. The molecular structure is shown in Fig. 1.

The sulphur atoms of Cys 41 and Cys 46 coordinate to one iron atom, and those of Cys 49 and Cys 79 to the other iron atom⁵. The coordination geometry around each iron atom is tetrahedral, judging from the electron density distribution around the atoms. The cluster is located near the molecular surface and is mainly surrounded by hydrophobic residues, leaving only one side, the sulphur atoms of Cys 41 and Cys 46, exposed to the solvent. In *P. aerogenes* ferredoxin and *Chromatium HiPIP*, electron transfer was suggested to be achieved through aromatic side chains in close contact with the [4Fe-4S] cluster^{3,10}. However, in *S. platensis* ferredoxin there are no invariant aromatic side chains in close contact with the [2Fe-2S] cluster. An alternative possibility is that electron transfer may occur via the orbitals of cysteinyl sulphur atoms, as these residues are directly bonded to the cluster and two of them are located outside the molecule, as described above. Comparison of the amino acid sequences of various chloroplast-type ferredoxins² shows that several ferredoxins, particularly those of higher plants, have deletions at positions 10 and 14 and, in this case, proline at position 11. The segment of residues 9-14 in *S. platensis* ferredoxin without the two deletions corresponds to the loop at the end of the antiparallel β -sheet. The overall structure of this region is probably very similar in the two types of ferredoxin, because the segment in ferredoxin without the two residues may take a 3_{10} turn by forming a hydrogen bond between the CO of residue 9 and NH of residue 13. In other words, the change in chain length of this region merely shifts the nature of turn without causing any major structural variation.

Figure 2 shows variations in the 26-amino acid sequence of chloroplast-type ferredoxins². The substitution frequencies of amino acids among 26 chloroplast-type ferredoxins are plotted against the distances between each α -carbon atom and the cluster, and Fig. 3 demonstrates that the carbon atoms distant from the cluster have higher substitution frequencies.

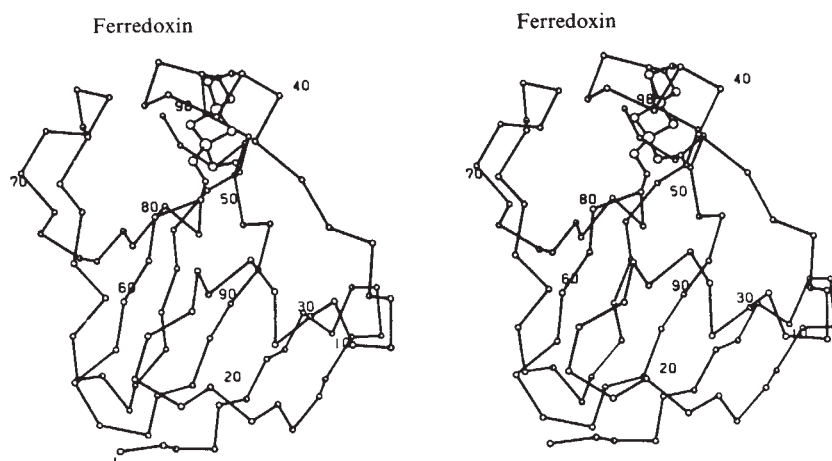
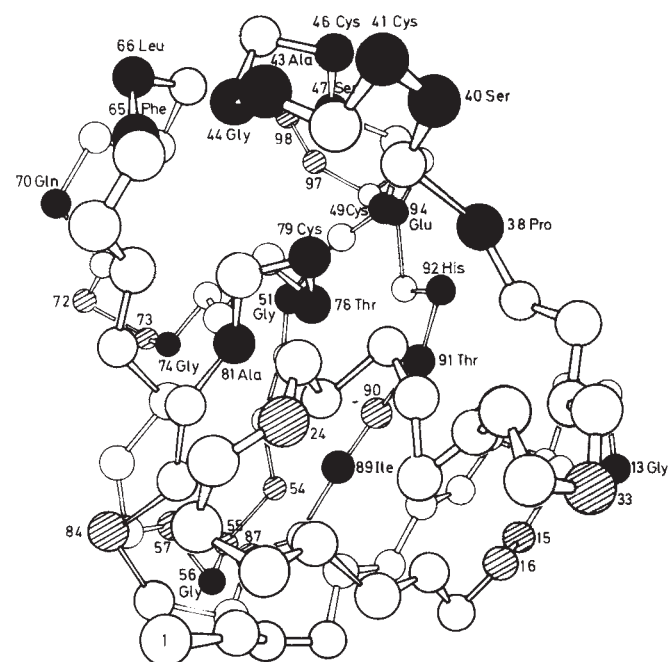


Fig. 1 The polypeptide chain of *S. platensis* ferredoxin shown by stereo pair drawing. The cluster is located at the top of the molecule. Residues 3-9 and 14-20 form an antiparallel β -sheet, and residues 5-8 and 87-89 form a parallel β -sheet. A short helical segment occurs at residues 25-31. There is a relatively large cavity in the interior of the molecule surrounded by the β -sheets and helix. The heavy-atom binding site is at Asp 36.



than 15 billion years ago. Some of the invariant glycine residues are located at the corners of the main chain: Gly 13, Gly 56 and Gly 74 must be necessary for chain folding.

Received 27 March; accepted 27 May 1980.

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Fig. 2 The amino acid sequence of *S. platensis* ferredoxin^{2,11} and the distribution of various amino acid residues present in chloroplast-type ferredoxins. The sequence of *S. platensis* ferredoxin (top row) is shown by the one-letter code. The different amino acid residues found in the sequences of 25 other chloroplast-type ferredoxins are shown below the sequence. —, Represents a deletion.

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Prevalence of amber suppressor-containing coliforms in the natural environment

Bonnie Marshall & Stuart B. Levy

Departments of Molecular Biology and Microbiology and of Medicine, Tufts University School of Medicine, Boston, Massachusetts 02111

Amber suppressible genes have been introduced into vectors^{1,2} and proposed for use in recombinant DNA studies. Such genes can be expressed only in a host bacterium with amber suppressors. To assess the biological containment of this kind of mutation we have now examined the frequency of bacteria with amber suppressors in faecal samples from humans and animals in different environments. This approach allowed us to evaluate the number of individuals in an environment bearing bacteria with amber suppressor mutations and the frequency of these bacteria within the faecal samples of each individual. Of all individuals sampled 30.5% contained detectable levels of bacteria with amber suppressor activity. In >75% of these samples, the level of suppressor-containing bacteria was <10%, about half of which were <1%. Most of these were *Escherichia coli*; however, *Citrobacter* and *Klebsiella* were also found. In three samples, essentially all coliforms had suppressor activity. Excluding these three samples, the estimated frequency of amber suppressors among faecal coliforms was 1.5%; however, if the data from these individuals were included, the overall frequency among faecal samples could be as high as 10%. By itself, the finding suggests that amber suppressor mutations in recombinant DNA vectors may not offer as great a safety feature as originally considered. These data, however, must be considered together with the known low sensitivity of wild-type *E. coli* to laboratory phages^{3,4} and their low recipient ability for some plasmids.

To perform these studies we required a genetic vector which could be introduced into wild-type host cells. Different phages proved unsuccessful. Because *incP1* plasmids have a wide host range, we chose this as a vector for our study. We were greatly aided by the availability of plasmid pLM2 (ref. 5) (developed by Leonard Mindich) which contains amber suppressible mutations⁶ in tetracycline and ampicillin resistance determinants while encoding a wild-type kanamycin resistance gene.

Samples of intestinal coliforms were obtained from five populations: hospital patients, laboratory workers, urban residents, rural residents and farm animals. A fresh faecal swab (Culturette; Scientific) was obtained from each donor, inoculated on to a MacConkey agar plate (Mac) and diluted by two-dimensional spreading on to four quadrants of the plates. These 'master' plates were then incubated at 37 °C overnight and refrigerated at 4 °C or tested immediately.

For mating studies with these coliforms, two *E. coli* strains were used as donors of plasmid pLM2: Ca274⁶ bearing pLM2 (from L. Mindich)⁵ and D15–12, a bile-salt sensitive, 'rough' *E. coli* produced in our laboratory by mating pLM2 into our host *E. coli* DO-19. The use of minimal agar eliminated the auxotrophic Ca274 from matings, while D15–12 was removed due to its death on the bile-salt-containing Mac agar.

The scheme for identification of endogenous suppressor-containing bacteria was as follows: a lawn of the donor organisms (Ca274 or D15–12) was inoculated on to a non-selective nutrient plate. The master plate, containing colonies of

coliforms from each faecal sample, was replica-plated⁷ on to the lawn of donor bacteria. After overnight incubation, the 'mating' plate was replica-plated on to media (minimal media for mating with Ca274; Mac agar for mating with D15–12) which would not allow the donor organism to grow and which contained either kanamycin (Kan) (40 µg ml⁻¹) or ampicillin (Amp) (50 µg ml⁻¹) + tetracycline (Tet) (10 µg ml⁻¹) + Kan (40 µg ml⁻¹).

The Kan replica plate assessed the efficiency of transfer of pLM2 to the wild-type coliforms; the Amp–Tet–Kan replica plate identified which of the recipient bacteria carried a suppressor mutation. Initially the master plate was replica-plated on to Mac+Kan (40 µg ml⁻¹) and on to Mac+Tet (10 µg ml⁻¹) + Amp (30 µg ml⁻¹) + Kan (40 µg ml⁻¹) to identify endogenous coliforms with resistance to Kan and/or resistance to Tet + Amp + Kan. Faecal samples bearing large numbers of bacteria resistant to Kan or a combination of Tet–Amp–Kan were excluded from analysis. pLM2 mating using either Ca274 or D15–12 showed 100% transfer efficiency of plasmid when known, good recipients were used in this replica-plating method. The observed frequency of transfer of pLM2 from both hosts was similar. Thus, the presence of any naturally occurring auxotrophic coliforms, which could have been missed in the Ca274 matings, was presumably small. The frequency of auxotrophic wild-type *E. coli* has been recently reported at about 9% (ref. 4).

Bacterial colonies, positive (Su⁺) and negative (Su⁻) for suppressor activity, were picked from the master plate, purified and retested by the plate method with the same or an alternative donor organism to confirm the presence or absence of amber suppression. Furthermore, some of the identified suppressor strains containing pLM2 were isolated and used to mate pLM2 into DO-11, an Su⁻, NaI^R strain (this laboratory) or into a Su⁺, streptomycin-resistant B40 (*supD*) strain. In all tests the intactness of the amber mutations on the plasmid was confirmed, with the exception of those few in which the presence of lytic phage prevented conjugation.

Faecal samples from 190 individuals from the five populations were tested. Of these, 30.5% had Su⁺ bacteria present, all among the lactose fermenters. Biotyping (Analytab, API 20E) of 36 Su⁺ isolates identified 33 as *E. coli*, 2 as *Citrobacter freundii* and 1 as *Klebsiella pneumoniae*. Most (>75%) of the faecal samples containing Su⁺ bacteria showed them at a frequency of <10%; in about 30% of samples, Su⁺ bacteria occurred even

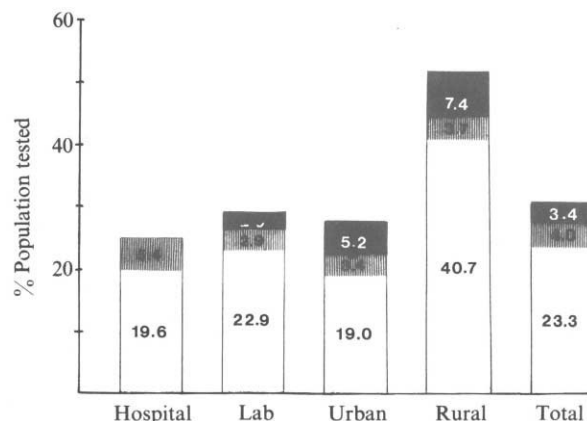


Fig. 1 Frequency of suppressor coliforms in faecal samples of four different human populations. The percentage of all samples containing any suppressor bacteria is represented by the whole bar. Frequencies (*f*) of suppressor occurrence within individual samples are shown by bar subdivisions as follows: open, 0 < *f* < 10%; hatched, 10 ≤ *f* < 50%; closed, *f* ≥ 50%. The number of individuals sampled from each population was: hospital, 56; laboratory, 35; urban, 58; rural, 27.

more rarely (<1.0%). Six samples showed a high level (>50%) of bacteria with suppressor activity (Fig. 1). Of note, we found three samples (from a laboratory worker and two urban dwellers) in which ~100% of the bacteria bore amber suppressors. Over 95% of the bacteria on these plates were recipients of the plasmid and Su^+ .

The frequency of Su^+ bacteria in faecal samples from males and females showed equal distribution both overall and within environments. Su^+ bacteria occurrence, however, was twice as common in the human rural population as in humans tested in the other three environments. Fourteen non-human faecal samples were also tested (six fowl, five cows, one fly, one goat, one kitten). Of these, four (28.6%) showed the presence of Su^+ bacteria (at levels of <10%). This is in keeping with the frequency found in samples from the total human population.

The frequency of Su^+ bacteria among the entire pool of faecal coliforms tested was estimated by dividing the approximately $3.5-5 \times 10^3$ Su^+ bacteria identified by the estimated $3.5-5 \times 10^4$ transconjugants on the 190 plates examined. This calculation gave an estimated frequency of Su^+ *E. coli* of ~10%. This figure, however, is elevated considerably by the three individuals whose samples were essentially all Su^+ . By excluding these individuals, the estimated frequency drops to roughly 1.5%.

The colonies that were not recipients of plasmid pLM2 obviously could not be evaluated for suppressor activity; however, there is no evidence to indicate that Su^+ bacteria have a different recipient ability from Su^- bacteria. Furthermore, the ability of the faecal bacterial colonies to mate with the test organism, in the conditions described, was generally high. In 40% of the samples, 90% or more of the colonies accepted pLM2, allowing a relatively complete examination of the available flora. In only 15% of the faecal samples were less than 20% of the total colonies able to receive the plasmid; in all cases, at least 10 colonies per sample were evaluable.

Our data show that lactose-fermenting bacteria with amber suppressor activity are not uncommon in nature, and seem to be more frequent than has been previously recorded. All were lactose fermenters, *E. coli* being predominant. Using the same plasmid, Robeson *et al.*⁴ found a level of about 0.4% suppressor-containing bacteria in 443 independently isolated wild-type *E. coli* from faecal and non-faecal sources. Our study, which examined whole faecal flora of different individuals, gave an estimated frequency of 1.5% in the majority of faecal samples. This value does not differ greatly from previous estimates⁴; however, the finding of 3 in 190 individuals with large amounts of Su^+ bacteria suggests that a much larger pool of these bacteria may exist in nature.

Given this frequency of Su^+ bacteria, the amber suppressor mutations in themselves, while providing some biological containment, may not be as effective as previously believed. The real effectiveness, however, must be considered in relationship to the actual ability of the mutant vector to propagate in wild-type coliforms. We and others have noted that some plasmids are poorly maintained in wild-type coliforms. In the case of λ vectors, the probability of vector rescue is greatly diminished because of the very low susceptibility of wild-type *E. coli* to this phage³. As an indication, we tested 27 isolates of our suppressor-containing coliforms with λ *vir*. None could be productively infected. Thus the degree of biological containment achieved by the introduction of suppressor mutations is greatly dependent on the choice of phage or plasmid vector used.

This study was supported by NIH contract no. NO1AI72529. We thank Toni Keller and Stanley Miller for help with the analyses.

Received 5 November 1979; accepted 23 April 1980.

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DNA cloning in *Streptomyces*: resistance genes from antibiotic-producing species

C. J. Thompson, J. M. Ward & D. A. Hopwood

John Innes Institute, Colney Lane, Norwich NR4 7UH, UK

The biochemical and morphological differentiation of actinomycetes makes them academically and economically interesting. Their secondary metabolites provide the majority of medically and agriculturally important antibiotics¹ (streptomycete genes may also be the primary source of clinically important antibiotic resistance²); their complex morphological developmental cycle involves a series of changes from vegetative mycelial growth to spore formation³. Recombinant DNA technology would add a powerful new dimension to the analysis of these various aspects of actinomycete biology and would also facilitate the development of industrial strains with increased antibiotic yield, or capable of making new antibiotics⁴. For most of these purposes, cloning of genes within and between actinomycetes is required to study the expression of particular genes in genetic backgrounds defined by mutations of the characters under study. To achieve this, we have now developed a method for molecular cloning involving the transfer of genes between unrelated streptomycetes.

For initial interspecific cloning experiments we chose *Streptomyces lividans* strain 66 (ref. 5) as host, since no restriction-modification systems are known in this strain and because a potential plasmid vector (SLP1.2) was available for it; all cleavage sites for the restriction endonucleases *Bam*HI and *Pst*I lie in a dispensable region of SLP1.2 (ref. 6). Into these sites we attempted to insert resistance genes from two antibiotic-producing streptomycetes. *Streptomyces fradiae*, which makes neomycin, contains a neomycin phosphotransferase (APH) and a neomycin acetyltransferase (AAC) (ref. 7 and J. Davies, personal communication). *Streptomyces azureus*, which makes thiostrepton, contains a ribosomal RNA methylase capable of protecting ribosomes from binding thiostrepton⁸.

Foreign DNA was inserted into the vector in two ways. The first approach used ligation of *Mbo*I genomic DNA fragments, of length 2-20 kilobase pairs, into the *Bam*HI site of SLP1.2 (ref. 9). *Mbo*I, which recognizes a sequence of only four base pairs (GATC), generates the same single-stranded termini (GATC) as *Bam*HI (recognition sequence GGATCC). The numerous *Mbo*I sites expected in *Streptomyces* chromosomal DNA (about 1 per kilobase pair; unpublished data) allowed us to generate a suitably random population of donor fragments by partial digestion with *Mbo*I (about 10% of the sites were cleaved). This seemed advisable as we knew nothing about the positions of restriction enzyme targets within genes or promoters, or about possible fragment size selection during ligation and transformation. The second approach used double digestion of SLP1.2 with *Bam*HI and *Pst*I to excise a 2.6-kilobase pair sequence of non-essential DNA and generate non-homologous single-stranded ends (one from the *Bam*HI and the other from a *Pst*I site). Recircularization required the insertion of a segment of DNA having corresponding termini. Such fragments were generated by partially cleaving donor chromosomal DNA with *Mbo*I to 5-28 kilobase pairs and then completely with *Pst*I (giving an average of one *Pst*I cut per *Mbo*I fragment).

Ligation mixtures containing SLP1.2 and fragments of *S. fradiae* or *S. azureus* total genomic DNA were transformed into *S. lividans* protoplasts. The original procedure¹⁰ was modified to increase the transformation efficiency approximately 20-fold. Transformation was indicated by the appearance of 'pocks' (circular zones of sporulation inhibition associated with plasmid transfer, sometimes called the *litz* (lethal zygotosis) phenotype¹¹ in the lawn of *S. lividans* growth arising from the regenerated

Table 1 Properties of the six recombinant plasmids

Strain	Plasmid	Size kilobase pairs		Method of construction		Presence of restriction fragment F	Site at vector-insertion junctions		Enzyme specified
		Plasmid	Insertion	Vector cleavage	Donor DNA cleavage		J1	J2	
M180	SLP1.2	14.4	0	—	—	+	—	—	None
TC7	pIJ1	24.8	13.0	<i>Pst</i> I/ <i>Bam</i> HI	<i>Pst</i> I/ <i>Mbo</i> I	—	Not <i>Bam</i> HI	<i>Pst</i> I	AAC
TC8	pIJ2	17.8	3.4	<i>Bam</i> HI	<i>Mbo</i> I	+	<i>Bam</i> HI	<i>Bam</i> HI	APH
TC9	pIJ3	22.1	10.3	<i>Pst</i> I/ <i>Bam</i> HI	<i>Pst</i> I/ <i>Mbo</i> I	—	Not <i>Bam</i> HI	<i>Pst</i> I	APH
TC10	pIJ4	23.4	11.6	<i>Pst</i> I/ <i>Bam</i> HI	<i>Pst</i> I/ <i>Mbo</i> I	—	Not <i>Bam</i> HI	<i>Pst</i> I	AAC
TC11	pIJ5	19.0	4.6	<i>Bam</i> HI	<i>Mbo</i> I	+	Not <i>Bam</i> HI	Not <i>Bam</i> HI	23S rRNA methylase
TC14	pIJ6	21.3	6.9	<i>Bam</i> HI	<i>Mbo</i> I	+	<i>Bam</i> HI	<i>Bam</i> HI	23S rRNA methylase

The recombinant plasmids were constructed by inserting genomic fragments containing genes for antibiotic resistance enzymes into SLP1.2. Neomycin resistance, due to AAC or APH, was cloned from *S. fradiae* (a variant of ATCC 10745 selected for high level resistance to neomycin and provided by Dr J. Davies); thiostrepton resistance (23S ribosomal RNA methylase) was cloned from *S. azureus* ATCC 14921. pIJ1, pIJ3 and pIJ4 were generated from *Pst*I and *Bam*HI cleaved SLP1.2 ligated to fragments of donor DNA double digested with *Mbo*I and *Pst*I; they therefore do not contain the small *Pst*I fragment F of SLP1.2 (see Fig. 3). pIJ2, pIJ5 and pIJ6 were constructed by ligation of SLP1.2 cleaved with *Bam*HI to *Mbo*I fragments of donor DNA. Plasmid and insert sizes (relative to the *Hind*III fragments of bacteriophage λ : 23.5, 9.59, 6.76, 4.44, 2.28, 1.94 and 0.58 kilobase pairs) were determined from the combined molecular weights of fragments in *Bam*HI/*Hind*III double digests, allowing for the presence or absence of fragment H (see Fig. 3). Insertion of DNA fragments having *Mbo*I termini into the *Bam*HI site of SLP1.2 reconstituted a *Bam*HI site in four out of nine hybrid junctions (J1 and J2 in Fig. 3) as deduced from the presence of fragments D and/or E (Fig. 3) in *Bam*HI/*Hind*III double digests (reconstitution was expected on random grounds in about one-third of junctions since one-half of each hexanucleotide junction contains half the original *Bam*HI sequence of the vector and the other half, coming from an *Mbo*I site in 73% G+C DNA, has a 36.5% ($73\% \times 0.5$) probability of having *Bam*HI specificity). The expected reconstitution of the *Pst*I site at J2 in plasmids pIJ1, pIJ3 and pIJ4 was confirmed by the presence of restriction fragment G (Fig. 3).

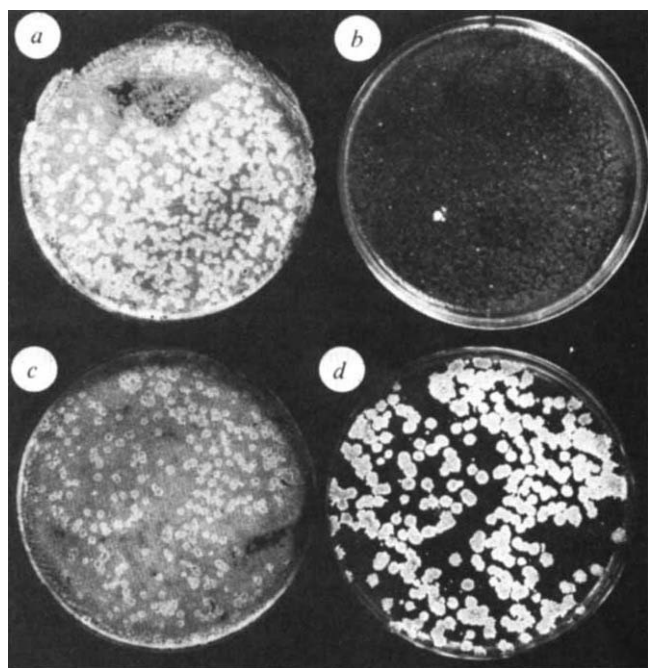


Fig. 1 The isolation of a clone carrying drug resistance. *S. azureus* genomic DNA (2.5 μ g) was partially cleaved with *Mbo*I into fragments of 2–20 kilobase pairs; SLP1.2 (0.5 μ g) was cleaved with *Bam*HI. Vector and genomic fragments were mixed and ligated (4 h at 10°C followed by 72 h at 4°C in ligation salts which contained 66 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM MgCl₂, 10 mM 2-mercaptoethanol and 0.1 mM ATP) at a total DNA concentration of 40 μ g ml⁻¹ with T4 DNA ligase (prepared according to ref. 16). Ligation products were introduced to 3×10^9 *S. lividans* strain 66 protoplasts (haemocytometer counts) by a modification (unpublished results) of the PEG-mediated transformation procedure¹⁰. These protoplasts gave rise to 5×10^7 colonies and 4×10^4 pocks (see a) after regeneration on 20 plates of R2 agar¹⁷ containing 0.5% Difco yeast extract (R2 YE). When these lawns were replica-plated to R2 YE plates containing 50 μ g ml⁻¹ thiostrepton, two spots of drug resistant growth appeared (one is shown in b). After purification of this strain, its plasmid DNA was isolated by CsCl-ethidium bromide centrifugation¹⁸ and used to re-transform *S. lividans* protoplasts. Non-selected regeneration (c) followed by replication to thiostrepton (d) demonstrated 100% correlation between pocks (c) and thiostrepton-resistant growth (d).

protoplast population (Fig. 1). When such lawns were replicated to plates containing antibiotic (50 μ g ml⁻¹ thiostrepton, or 1 μ g ml⁻¹ neomycin), patches of growth appeared corresponding to certain pocks on the regeneration plates (2 thiostrepton-resistant out of 40,000 pocks in an experiment with *Mbo*I-generated fragments, 2 neomycin-resistant out of 10,000 pocks in a similar experiment, and 3 neomycin-resistant out of 10,000 pocks when the donor DNA was cleaved with *Mbo*I and *Pst*I). The drug resistance and liz phenotypes of these clones were transferred coincidentally at high frequency (10–100%) in matings with genetically marked *S. lividans* derivatives, indicating that they contained drug resistance genes inserted into SLP1.2. This was confirmed by transformation using the CCC plasmid DNA which could be isolated from each clone; the plasmids conferred drug resistance (Fig. 1) on all (>95%) transformants (that is, pocks).

Agarose gel electrophoresis of the hybrid plasmids revealed that each was larger than SLP1.2 (Fig. 2). *Hind*III digestion of each plasmid yielded SLP1.2 fragments B and C, as expected (Fig. 3). *Hind*III fragment A, which contained the cloning sites, was, however, invariably increased in size (Fig. 2). Since the inserted fragments came from DNA which had been only partially digested with *Mbo*I, they could not be excised in one piece with *Mbo*I. Moreover, ligation of *Streptomyces* (73% G+C) *Mbo*I fragments into a *Bam*HI site should alter the sequence at about two-thirds of the *Bam*HI-*Mbo*I junctions to eliminate the *Bam*HI site. The fact that a *Bam*HI recognition site was found at four out of nine recombinant junctions (Table 1) confirmed this prediction, and will allow the precise removal of the neomycin-(APH) and thiostrepton-resistance genes from the recombinant plasmids pIJ2 and pIJ6 for recloning purposes. The increased size of *Hind*III fragment A was used to calculate the amount of inserted DNA in each clone (Table 1). The sizes of the inserts (4–13 kilobase pairs) agreed with the molecular weight range of the donor DNA fragments.

Interspecific gene transfer was confirmed biochemically. The four neomycin-resistant clones were assayed for APH and AAC activities; two contained APH and two AAC. Extracts from both thiostrepton-resistant clones were found to contain thiostrepton-resistant ribosomes and the expected ribosomal RNA methylase activity. Thus the availability of *S. lividans* clones containing the ribosomal RNA methylase of *S. azureus* confirmed its potential to confer thiostrepton resistance on the producing organism⁸. In the case of neomycin resistance, the *S. lividans* clones established that the presence of either of the two neomycin modifying enzymes of *S. fradiae* is sufficient to give

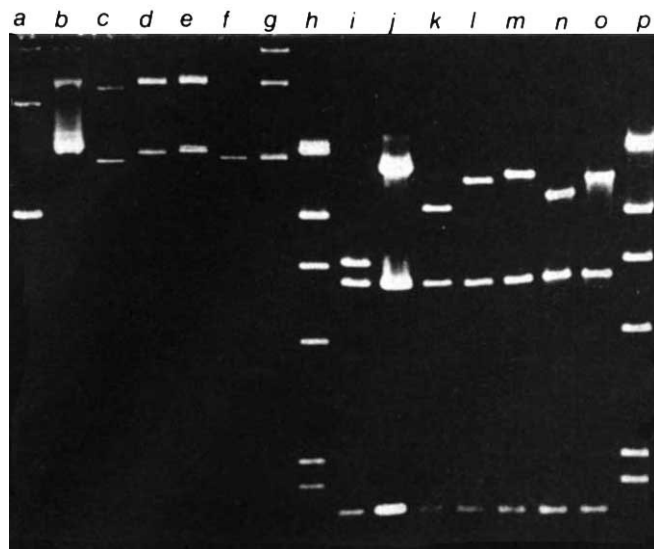


Fig. 2 Agarose gel electrophoresis of recombinant plasmids and their cleavage fragments produced by *Hind*III. The DNA species were separated by electrophoresis in 0.8% agarose using a Tris-acetate buffer¹⁹ for 15 h at 1 V cm⁻¹. *h* and *p*, bacteriophage λ DNA cleaved with *Hind*III; *a*, the parent SLP1.2 plasmid and *b-g*, the recombinant plasmids pIJ 1-6 respectively, uncleaved (the fastest migrating band in each track is the CCC plasmid species); *i*, SLP1.2 and *j-o*, pIJ1-6 respectively, cleaved with *Hind*III. Note the indistinguishable sizes of the two smaller *Hind*III fragments in SLP1.2 and the series of recombinant plasmids and the increased size of the largest fragment in each recombinant.

resistance to neomycin. Comparable clones promise to be valuable in understanding the resistance mechanisms of other antibiotic-producing actinomycetes and perhaps the origins of clinically important drug resistance genes².

The results, in conjunction with those of Bibb *et al.*¹² on the cloning of methylenomycin resistance from *S. coelicolor* into *S. coelicolor* and *S. lividans*, and our unpublished results showing that two randomly chosen genes for amino acid biosynthesis could be cloned from the chromosome of *S. coelicolor* A3(2) on to the SCP2* plasmid of that strain, indicate that molecular cloning in streptomycetes on existing plasmid vectors is quite practicable. We feel that our cloning of three genes conferring

resistance, by known mechanisms, to stable and readily available antibiotics augurs well for the further development of cloning vectors for streptomycetes. Possible resistance enzymes for many aminoglycosides⁷, macrolides¹³, chloramphenicol¹⁴ and other antibiotics are available in the actinomycetes. It should therefore be relatively straightforward to build a streptomycete vector bearing more than one resistance gene in order to: select for the presence of the vector¹⁵, detect clones by insertional inactivation¹⁵, and achieve high level expression of cloned genes (highly efficient promoters for some of these genes are being sought among mutant strains showing increased drug resistance). Such a vector would facilitate, for example, the cloning of genes involved in antibiotic biosynthesis or differentiation, on which this laboratory is now embarked.

We thank Dr Eric Cundliffe for the *S. azureus* strain and the RNA methylase assays, Dr Ken-ichi Komatsu for the assays of AAC and APH, Drs Keith Chater, Julian Davies and Tobias Kieser for helpful discussions, and the National Research Development Corporation for financial support.

Received 20 March; accepted 21 May 1980.

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DNA cloning in *Streptomyces*: a bifunctional replicon comprising pBR322 inserted into a *Streptomyces* phage

J. E. Suarez & K. F. Chater

John Innes Institute, Colney Lane, Norwich NR4 7UH, UK

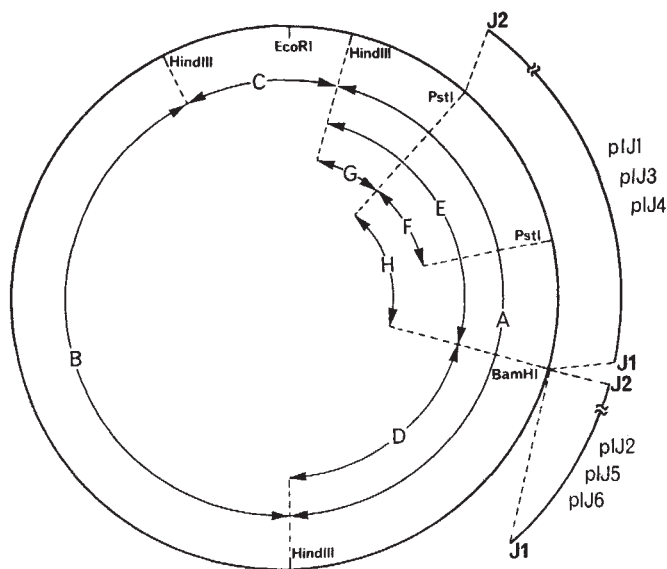


Fig. 3 Restriction map of the SLP1.2 plasmid for enzymes *Eco*RI, *Hind*III, *Pst*I and *Bam*HI⁶ (continuous circle) and positions of insertions in recombinant plasmids pIJ1-6 (Table 1).

The Gram-positive, mycelial, differentiating streptomycetes are responsible for the production of many important antibiotics. The availability of gene cloning systems in this microbial group would have many industrial applications¹ besides allowing more penetrating study of the genetics of *Streptomyces coelicolor* A3(2) (which, as the best understood streptomycete genetically, serves as a model for much other *Streptomyces* genetics)². Recent successes^{3,4} (see previous paper⁴) in introducing *Streptomyces* DNA into *S. coelicolor* and *Streptomyces lividans* on plasmid vectors would be nicely complemented by the availability of *Streptomyces* bacteriophage vectors (discussed in ref. 5): for example, many phages have wide and easily defined host ranges; heat-inducible prophages might be used to give high copy number of cloned DNA; efficient phage promoters might be used to increase gene expression; there may be differential stabilities for particular DNA sequences cloned in plasmids *vis-à-vis* phages; selective insertion of DNA, utilizing packaging constraints, may be possible with phages; and *in situ* hybridization⁶ of radioactive probes to DNA in plaques is likely to be simple. We describe here the use of the moderately wide host range temperate phage, Φ C31⁷, for this purpose.

Although Φ C31 DNA (39 kilobases) is not known to have single targets for any restriction enzymes, it possesses six *Eco*RI sites⁵ which might be used for inserting DNA. Viable deletion mutants have been obtained⁵ that should allow the insertion of fragments of foreign DNA without interfering with packaging of DNA into maturing phage particles. DNA of Φ C31 efficiently transfects *Streptomyces* protoplasts in the presence of polyethylene glycol⁸. The particular phage strain chosen was Φ C31 Δ 23, which has about 8% (3.25 kilobases) less DNA than the wild-type Φ C31 (J.E.S. and K.F.C., unpublished observations). The small *Escherichia coli* plasmid pBR322 (4.362 kilobases)^{9,10}, which has single *Eco*RI, *Bam*HI and *Pst*I sites, was selected as the DNA for insertion.

The Φ C31 Δ 23 vector DNA was treated¹¹ with T4 ligase to close its cohesive ends covalently^{5,12}, precipitated with ethanol and partially cleaved with *Eco*RI to give an average of about one cut per molecule. After a second ethanol precipitation, the DNA was ligated in the presence of a 25-fold molar excess of completely *Eco*RI-cleaved pBR322, and the DNA mixture was used to transfect *S. lividans* protoplasts⁸. Of 1,500 plaques obtained with a sample of DNA mixture containing 100 ng Φ C31 Δ 23 DNA (about 30-fold fewer plaques than expected for 100 ng Φ C31 *cts* DNA in a normal transfection)⁸ one hybridized to³²P-labelled pBR322 probe DNA in a filter-hybridization test⁶.

DNA was isolated from the purified presumed hybrid phage and analysed by digestion with restriction enzymes (Fig. 1). An *Eco*RI fragment of 4.36 kilobases, present in the hybrid DNA but not in DNA of Φ C31 Δ 23, co-migrated with *Eco*RI-cleaved pBR322. Single sites for *Bam*HI and *Pst*I, absent from

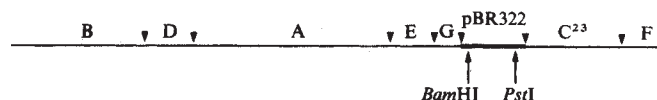


Fig. 2 Structure deduced for Φ C31 Δ 23-pBR322 hybrid DNA from data in Fig. 1, based on the *Eco*RI restriction map of Φ C31 DNA given in ref. 17. Letters above the map are *Eco*RI fragment designations.

the control DNA, were present in the hybrid DNA, and were both within the novel *Eco*RI fragment (not shown). In digestions with single enzymes, the smaller *Bam*HI fragment was 10.8 kilobases, and the smaller *Pst*I 7.4 kilobases. These data were consistent with insertion of pBR322 into Φ C31 Δ 23 in the position and orientation shown in Fig. 2.

Wild-type Φ C31 DNA contains no sites for the *Pst*I isoschizomer *Sal*PI present in *Streptomyces albus* P: consequently, Φ C31 is not restricted by *S. albus* P¹³. However, phage from the plaque containing pBR322 sequence homology was restricted and modified by *S. albus* P: its efficiency of plating on *S. albus* P was 20-fold lower (data not shown) when the previous host was *S. lividans* than when it was *S. albus* P (using *S. lividans* as a standard host). A similar level of restriction by *S. albus* P was previously seen with phage R4, which has one *Sal*PI target site in its DNA¹⁴. Presumably, introduction of the *Pst*I (= *Sal*PI) site of pBR322 into Φ C31 was responsible for the observed restriction. This property provided a supplementary way of identifying the hybrid clone.

The *Eco*RI site of pBR322 is not within a region needed for plasmid replication or expression of its tetracycline or ampicillin resistance genes in *E. coli*. Therefore, assuming that the Φ C31DNA would be largely unexpressed in *E. coli*, the hybrid molecule should replicate in *E. coli* as a plasmid and express both drug resistances. This was found to be the case: very similar efficiencies of transformation¹¹ of a *recA* derivative of *E. coli* 803 (*metB hsdS*) to tetracycline resistance were obtained with supercoiled pBR322 DNA and ligated or non-ligated hybrid DNA. (The similarity between the efficiencies with ligated and non-ligated DNA probably reflects the high stability of hydrogen bonding between the cohesive ends of Φ C31 DNA^{5,12}.) The colonies obtained were all Met⁻ and resistant to both tetracycline (25 μ g ml⁻¹) and ampicillin (200 μ g ml⁻¹) when tested by replica plating. Plasmid DNA isolated from nine colonies transformed with hybrid DNA gave an *Eco*RI digestion pattern which was distinguishable from that obtained with the original hybrid DNA isolated from phage particles only by the expected covalent joining of the cohesive ends of the phage DNA (Fig. 1). (Plasmid DNA isolated from two of the *E. coli* colonies transformed with pBR322 DNA was indistinguishable from pBR322 using *Eco*RI digestion.)

We next tested whether *S. lividans* protoplasts could be transfected by the hybrid plasmid DNA isolated from *E. coli*. About 250 ng hybrid plasmid DNA gave 1,100 plaques—a frequency 10–100-fold lower than that obtained with DNA isolated from the hybrid phage. DNA from phages obtained from five of these plaques was indistinguishable by restriction enzyme analysis from the DNA of the original hybrid (Fig. 1).

The data reported here demonstrate the following: (1) the plaque hybridization technique⁶ is applicable to the *Streptomyces* phage Φ C31; (2) pBR322 DNA can be inserted into Φ C31 Δ 23 DNA at an *Eco*RI site 6.35 kilobases from one end of the molecule without obvious effects on the ability of the phage to form plaques, proving the feasibility of using this phage as a DNA cloning vector and providing single dispensible *Bam*HI and *Pst*I target sites which will be important in the future use of derivatives of the hybrid as cloning vectors; (3) the presence of the *Pst*I (= *Sal*PI) site of pBR322 in the hybrid phage causes it to be restricted by *S. albus* P; (4) DNA of the Φ C31 Δ 23-pBR322 hybrid can easily be transformed into *E. coli* (even without ligation of the phage cohesive ends), and maintained there without any evidence of structural instability

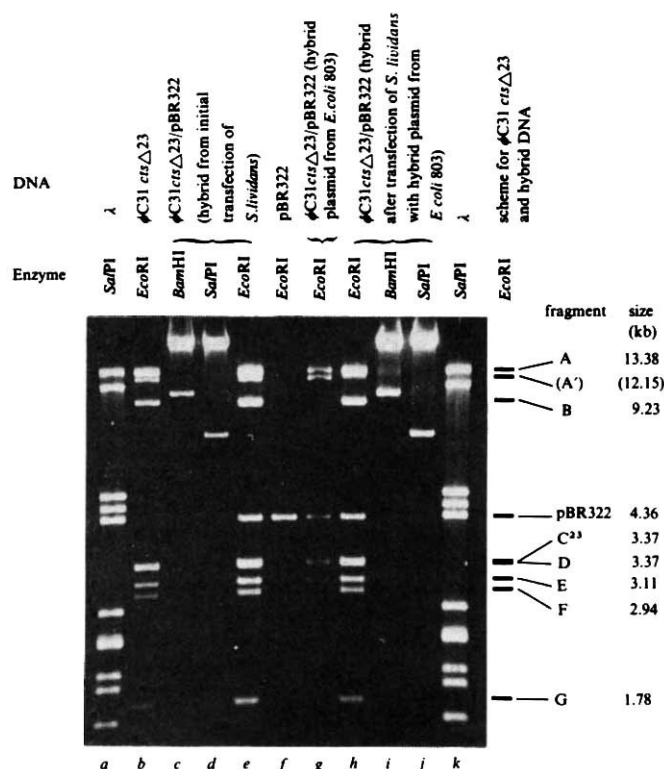


Fig. 1 Restriction enzyme analysis of DNA of Φ C31 derivatives using electrophoresis in 1% agarose gels. Tracks a and k are molecular size standards of λ DNA treated with *Sal*PI (ref. 13). Phage DNA was prepared by conventional procedures (ref. 16). Note that the DNA in tracks c, d and e is indistinguishable from that in tracks i, j and h, but that (as expected) in the plasmid form of the hybrid DNA (track g), *Eco*RI fragment A' (the fusion product of *Eco*RI fragments B and F, the cohesive ends of the phage DNA⁵) completely replaces B and F. Fragment C²³ (3.4 kilobases (kb)) is the reduced form present in Φ C31 Δ 23, of the wild-type fragment C (6.6 kilobases, absent from this gel) (ref. 5).

during subculture for at least 30 cell generations; (5) the supercoiled covalently closed circular DNA isolated from *E. coli* transfects *S. lividans* protoplasts at least moderately efficiently, suggesting the absence of a severe restriction barrier (which might have operated on DNA last propagated in *E. coli*); (6) the pBR322 sequence is stably inherited during propagation of the hybrid molecule as a phage in *S. lividans*.

The ability of the hybrid DNA to inhibit either *Streptomyces* or *E. coli* will be invaluable in introducing some of the powerful genetic techniques (such as transposon mutagenesis)¹⁵ available in *E. coli* into the study of *Streptomyces* genetics. It may also be possible to turn to advantage the absence of packaging constraints on the size of the molecule when it is replicating in the plasmid mode, *vis-à-vis* the presence of such constraints during its growth as a phage.

Dr A. G. Hepburn provided not only extensive technical advice but also labelled and unlabelled pBR322 DNA. We thank Professor D. A. Hopwood and Dr C. J. Thompson for invaluable comments on the manuscript, and Celia Bruton for technical assistance. J.E.S., on leave from the University of Oviedo, Spain, was the recipient of a grant from the Vicente Canada Blanch Foundation.

Received 19 March; accepted 21 May 1980.

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Novobiocin inhibition of simian virus 40 DNA replication

Howard J. Edenberg

Department of Biochemistry, Indiana University School of Medicine, Indianapolis, Indiana 46223

Supercoiling in eukaryotic cells can be explained by the packaging of DNA into nucleosomes in the presence of nicking-closing enzymes¹. This does not, however, rule out the presence of a DNA gyrase. DNA gyrase² is involved in the introduction and maintenance of supercoiling in prokaryotic cells^{3–6} and in DNA replication in *Escherichia coli*^{4,6,7}. Novobiocin and coumermycin-A₁ inhibit prokaryotic DNA replication^{4,7–11} by inhibiting the subunit of the gyrase responsible for its ATPase and energy transduction activities^{3,4,12–14}. The inhibition of DNA synthesis in a mammalian cell¹⁵ and in rat liver mitochondria¹⁶ by novobiocin suggests that a gyrase required for initiation of replication is inhibited. Because the presence of a gyrase in eukaryotic cells would be of great interest, I have examined this question in a well characterized model system, the replication of simian virus 40 in its host (monkey) cell. The results demonstrate here that novobiocin and coumermycin A₁ inhibit SV40 DNA synthesis in mammalian cells and inhibit DNA polymerase α , the replicative DNA polymerase in this system^{17,18}. Novobiocin, but not coumermycin A₁, reduces the degree of supercoiling of DNA pulse-labelled in its presence, probably through its inhibition of protein synthesis. Thus the inhibition of SV40 DNA synthesis by novobiocin does not alone prove the involvement of a DNA gyrase.

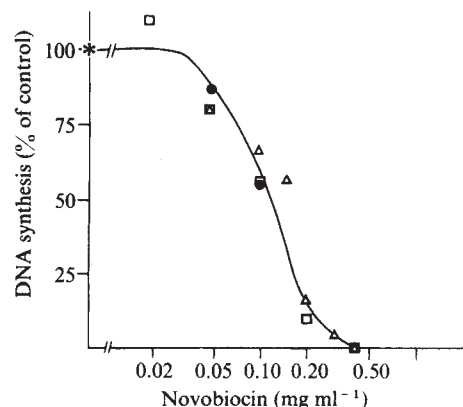


Fig. 1 Novobiocin inhibition of SV40 DNA synthesis in intact cells. CV-1 cells were grown to confluence in minimal essential medium (MEM) with 10% fetal calf serum (FCS), infected with SV40, and incubated in MEM with 2% FCS as previously described²⁰. Novobiocin (Boehringer) was added from a stock solution of 10 mg ml⁻¹ in 2.0 mM Tris-HCl, pH 7.5, as noted. Radioactive labelling was terminated by removal of medium, two rinses with phosphate buffered saline, and extraction of viral DNA by the method of Hirt¹⁹. □, Novobiocin was added 20 h after infection, [³H]dT (1.8 µCi ml⁻¹) was added 75 min later, and incubation was continued for a further 24 h. △, Novobiocin was added 18.5 h after infection, [¹⁴C]dT (0.08 µCi ml⁻¹) was added 90 min later and incubation was for a further 25 h. ●, At 39 h after infection, the medium was replaced with fresh medium containing 2% FCS and 12.5 mM HEPES (pH 7.1); 2 h later novobiocin was added, and 120 min after drug addition [³H]dT (12 µCi ml⁻¹) was added for a 30-min pulse.

The effect of novobiocin on SV40 DNA synthesis was studied by examining the incorporation of radioactive label into viral DNA (selectively extracted from the cells by the method of Hirt¹⁹ about 40 h after infection). As shown in Fig. 1, novobiocin inhibits SV40 DNA replication in a dose-dependent manner; 50% inhibition is achieved by ~0.1–0.2 mg ml⁻¹ novobiocin. Inhibition of DNA synthesis by novobiocin is rapid (the steady-state level is reached within 90 min) and reversible. The concentration of novobiocin required to inhibit SV40 replication is about 100 times greater than that required to inhibit DNA synthesis in *E. coli*^{7,9}.

Since the inhibition of processes in two different systems by the same drug does not necessarily imply that the same target enzyme is involved in both systems, I tested the effect of novobiocin on the replicative DNA polymerase. We have previously shown that DNA polymerase α is responsible for SV40 DNA synthesis^{17,18}. DNA polymerase α , partially purified from the host monkey cells, was tested in a standard *in vitro* assay¹⁷ and as shown in Fig. 2 was 50% inhibited by ~0.2 mg ml⁻¹ novobiocin, a concentration similar to that which inhibits replication in cells. This inhibition would suffice to explain the inhibition of SV40 DNA synthesis. Since the substrate in these studies consists of short linear segments of DNA which are extensively nicked and gapped, this inhibition cannot be related to a need to relieve supercoiling. DNA polymerase γ is also inhibited (50% at about 0.6 mg ml⁻¹); DNA polymerase β is not inhibited (data not shown). These results confirm reports that similar concentrations of novobiocin inhibit a crude DNA polymerase α of rat brain²¹ and that high levels inhibit a human 7S DNA polymerase²², but do not significantly inhibit DNA polymerase β ^{21,22}.

Closed circular SV40 DNA made during a 30–40 min pulse in novobiocin-inhibited cells (0.1 mg ml⁻¹ and above) has a greater density in CsCl-ethidium bromide density gradients than does SV40 DNA from control cells (Fig. 3), indicating that it is less negatively supercoiled^{23,24}. This was confirmed by examining the dependence of sedimentation rate on ethidium bromide

concentration²³⁻²⁵; the minimum sedimentation rate of SV40 DNA extracted from control cells occurred at about $2 \mu\text{g ml}^{-1}$ ethidium bromide, while that of SV40 DNA made in the presence of $0.1\text{--}0.2 \text{ mg ml}^{-1}$ novobiocin occurred at about $1.0\text{--}1.5 \mu\text{g ml}^{-1}$. Novobiocin itself does not bind to DNA⁸, and would not directly alter the supercoiling. DNA made during long labelling periods in the presence of novobiocin is indistinguishable from that made in control cells (data not shown). At these levels of novobiocin, protein synthesis is also inhibited (data not shown) and the change in supercoiling could be a result merely of less packaging of the DNA into nucleosomes due to this inhibition of protein synthesis.

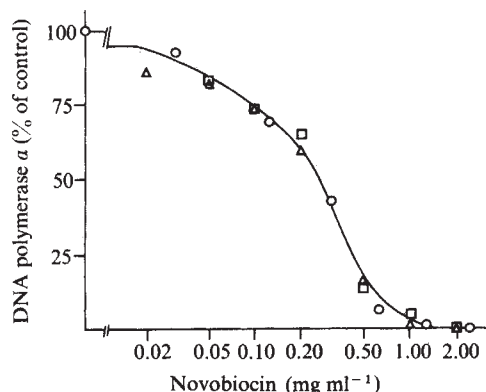


Fig. 2 Novobiocin inhibition of DNA polymerase α *in vitro*. DNA polymerase α assays were conducted as previously described¹⁷; the reaction mixtures contained (per 0.1 ml) $50 \mu\text{g}$ activated DNA and $15 \mu\text{g}$ bovine serum albumin, in 0.05 M Tris-HCl pH 8.5, 7.5 mM MgCl_2 , all four dNTPs at either $15 \mu\text{M}$ ($\circ$, Δ) or $25 \mu\text{M}$ ($\square$); and [^3H]dTTP ($2 \mu\text{Ci}$). Two different preparations of DNA polymerase α were used, one partially purified through DEAE cellulose as previously described¹⁷ ($\circ$) and an independent preparation purified in a similar manner and then further purified through phosphocellulose²⁶ (Δ , $\square$). Both preparations were shown to be sensitive to *N*-ethyl maleimide, and resistant to dideoxy-TTP^{17,18,27}. Incorporation was normalized to the value from reactions lacking drugs; 100% represents 235 pmol ($\circ$), 340 pmol (Δ), or 180 pmol ($\square$) of dTTP incorporated in 30 min.

To clarify this situation, I examined the effects of coumermycin- A_1 (refs 10, 11) on SV40 DNA synthesis. Coumermycin- A_1 lyses monkey cells at concentrations above 0.01 mg ml^{-1} ; at slightly lower concentrations it inhibits SV40 DNA replication, and at slightly higher concentrations inhibits DNA polymerase α (50% at about 0.03 mg ml^{-1}) and γ (50% at 0.2 mg ml^{-1}). Coumermycin- A_1 , however, inhibits DNA replication without significantly altering the supercoiling of SV40 DNA, as tested both with equilibrium density gradients (Fig. 3c) and sedimentation rate measurements in the presence of ethidium bromide. These comparative data reinforce the suggestion that the difference in supercoiling seen with novobiocin is a secondary effect of the drug.

After submission of this manuscript, the presence of type II DNA topoisomerases in eukaryotic cells was reported²⁸; no gyrase activity was reported. These topoisomerases are reported to be sensitive to high levels of novobiocin²⁸, and the authors relied on the previously reported sensitivity of replication to high levels of novobiocin¹⁵ to argue that these topoisomerases might be the target enzymes. These conclusions are premature, since the data presented here demonstrate that the effects of novobiocin and coumermycin- A_1 on SV40 DNA synthesis can be explained as the results of inhibiting DNA polymerase α and protein synthesis. The results presented here provide no direct

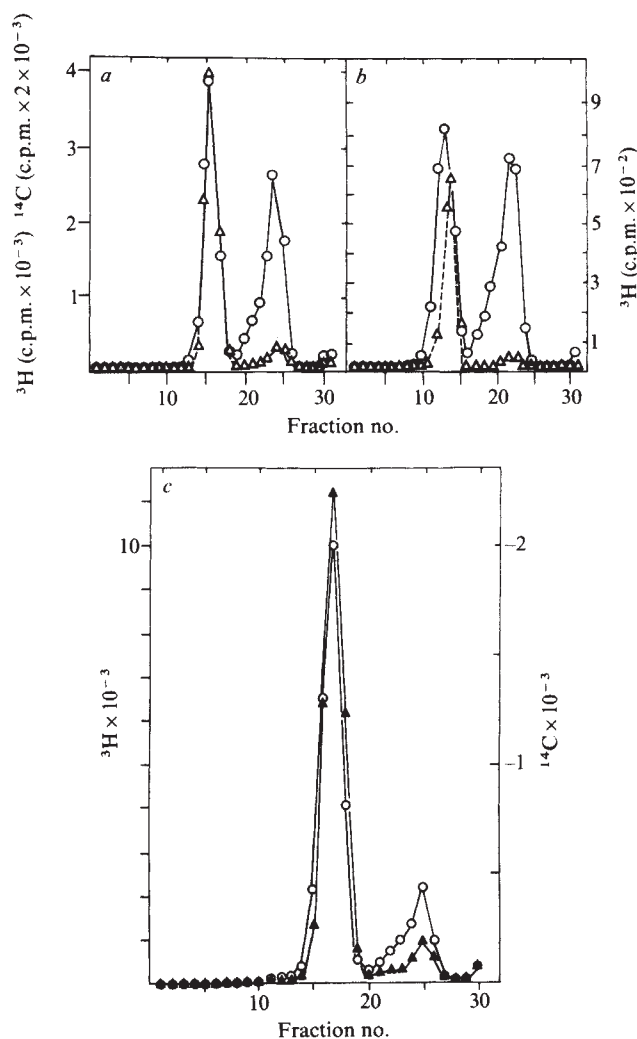


Fig. 3 Ethidium bromide-caesium chloride equilibrium gradients. Control SV40 DNA was isolated by the method of Hirt¹⁹ from infected cells incubated with [^{14}C]dT ($0.08 \mu\text{Ci ml}^{-1}$) from 22 to 45 h post-infection (Δ). Pulse-labelled DNA was isolated in the same manner at 40 h post infection ($\circ$). *a*, Cells were incubated for 20 min with [^3H]dT ($25 \mu\text{Ci ml}^{-1}$), in the absence of drug. *b*, Cells were incubated for 2 h with 0.2 mg ml^{-1} novobiocin, and then [^3H]dT ($25 \mu\text{Ci ml}^{-1}$) was added for 35 min. *c*, Cells were incubated for 2 h with 0.005 mg ml^{-1} coumermycin- A_1 and then [^3H]dT ($25 \mu\text{Ci ml}^{-1}$) was added for 40 min. The Hirt supernates were extracted with chloroform/isoamyl alcohol (24:1), aliquots of ^{14}C -DNA (control) (Δ) were mixed with the ^3H -DNA (pulse) ($\circ$) from control or drug-treated cells, and each was adjusted to a refractive index of 1.388 with CsCl , buffer (0.01 M Tris-HCl pH 8.0, 0.01 M NaCl, 0.001 M EDTA) and ethidium bromide (0.27 mg ml^{-1} final concentration). Centrifugation was for 42 h at $38,000 \text{ r.p.m.}$, 20°C in a Beckman 50Ti rotor. Fractions were collected, carrier DNA was added, and the DNA precipitated with HCl and filtered through glass fibre filters, all as previously described²⁰. For *a* and *b*, ^{14}C scales are the same, ^3H scales differ. Density decreases from left to right.

evidence for participation of a DNA gyrase in SV40 DNA replication, although they do not rule out the possibility.

This research was supported by a USPHS grant R01 GM 25681.

Received 20 February; accepted 26 May 1980.

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Identification of integrated hepatitis B virus DNA and expression of viral RNA in an HBsAg-producing human hepatocellular carcinoma cell line

Prasanta R. Chakraborty, Nelson Ruiz-Opazo, Daniel Shouval* and David A. Shafritz

Departments of Medicine and Cell Biology and the Liver Research Center, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

Alexander and co-workers¹ have developed a tissue culture cell line (PLC/PRF/5) from a primary hepatocellular carcinoma of a Mozambican male with a positive serum for hepatitis B virus surface antigen (HBsAg). This cell line produces and secretes HBsAg but no other known viral protein¹⁻³. PLC/PRF/5 therefore contains at least part of the hepatitis B virus (HBV) genome in functional form. The 42-nm, spherical Dane particle in human serum is thought to represent the complete infectious agent of HBV⁴. The core of this particle contains circular double-stranded DNA in which one strand is 'nicked' (complete but not covalently closed) and the other strand is incomplete (lacking 15-45% of its sequence complement)^{4,5}. Four proteins have been associated with the HBsAg, core antigen (HBcAg), e antigen and a viral DNA polymerase⁴. Several groups have now cloned Dane particle DNA in *Escherichia coli*⁶⁻¹⁰, and both restriction maps and a complete DNA sequence have been determined. Various studies suggest that there is sequence heterogeneity in Dane particle DNA isolated from patients expressing the same or different viral subtypes, as well as sequence variation in different clones of Dane particle DNA obtained from a given individual^{10,11}. Other studies^{12,13} have reported HBV DNA sequences in DNA from liver cancer tissue. In none of these cases was viral DNA reported to be integrated into the host cell genome. With the improvement in techniques for characterizing viral DNA molecules, we have begun to analyse HBV genes and their expression in the PLC/PRF/5 cell line by molecular hybridization of cellular DNA and RNA to cloned ³²P-labelled plasmid HBV DNA. We now present evidence in PLC/PRF/5 cells for integration of HBV DNA into the host genome and expression of three RNA molecules containing HBV-specific sequences. These findings are consistent with observations in several animal virus models in which integrated viral DNA is found during oncogenic transformation.

HBV DNA contains a unique *Eco*RI site which was used for insertion of HBV sequences. Therefore, treatment of cloned recombinant λ phage or plasmid pA01 DNAs containing the complete sequence of fully double-stranded Dane particle DNA (3,250-3,300 base pairs) with *Eco*RI should release the entire sequence of HBV DNA as a single fragment, which can be identified by agarose slab gel electrophoresis, nitrocellulose filter transfer and hybridization to an appropriate ³²P-labelled

HBV probe (Southern blot)¹⁴. Figure 1 shows the autoradiograph of a Southern blot of recombinant λ HBV DNA, λ HBV DNA digested with *Eco*RI and λ HBV DNA digested with *Eco*RI followed by a second restriction endonuclease to generate additional HBV fragments. *Eco*RI generates a single HBV fragment of ~3,250 base pairs (lane b), *Eco*RI followed by *Bam*HI generates two fragments of ~1,900 and 1,400 base pairs (lane c), *Eco*RI plus *Bgl*II generates three fragments of ~2,250, 700 and 100 base pairs (lane d), *Eco*RI plus *Hinc*II yields four fragments of ~1,800, 800, 450 and 200 base pairs (lane e) and *Eco*RI plus *Hind*III (lane f) generates one 3,250-base pair fragment as found with *Eco*RI alone (that is, the HBV sequence in recombinant λ phage DNA contains no *Hind*III restriction site). In each case, the total length of HBV restriction fragments is approximately 3,250 base pairs. For reasons which are unclear, in lane e the hybridization signal for the 1,800 base pair band was weak and this band reproduced poorly on the photograph. For subsequent studies, restriction enzymes *Eco*RI and *Hind*III were used.

Figure 2 shows the autoradiograph of a Southern blot of PLC/PRF/5 DNA digested with either *Eco*RI or *Hind*III, using

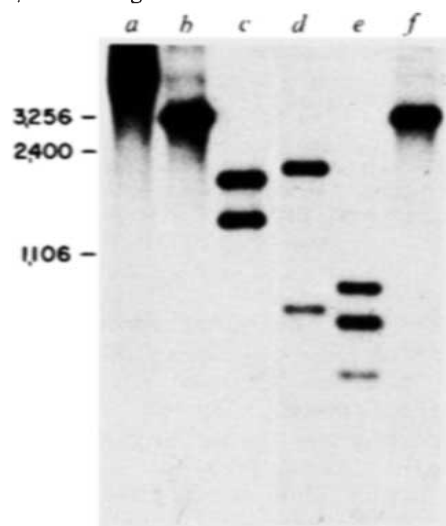


Fig. 1 Restriction enzyme analysis of cloned λ HBV DNA. Recombinant (5 μ g) λ HBV DNA was digested with 5 units of *Eco*RI restriction endonuclease. Digested material (1 μ g) was further digested with 1 unit of *Bam*HI, *Bgl*II, *Hinc*II or *Hind*III, respectively. All digestions were carried out in appropriate buffers at 38°C for 6-12 h. Aliquots (1 μ g) of each digested sample were placed on a separate lane of a 0.8% agarose slab gel and electrophoresis carried out in Peacock and Dingman buffer³¹ at 50 V for 5-6 h. After electrophoresis, the DNA fragments were transferred to nitrocellulose paper and the paper was hybridized with nick-translated ³²P-labelled pA01 HBV probe as described by Southern¹⁵. Nick-translation was carried out according to a modified procedure of Rigby *et al.*, using *E. coli* DNA polymerase I. Both ³²P-dATP and ³²P-dCTP (specific activity 300-800 Ci mmole⁻¹) were included in the reaction to obtain uniform labelling of high specific activity (1-2 $\times 10^8$ per DNA). After hybridization, the nitrocellulose paper was exposed overnight on Kodak XR-5 film for autoradiography. Lane a, undigested λ HBV DNA, lane b, λ HBV DNA + *Eco*RI; lane c, λ HBV DNA + *Eco*RI + *Bam*HI; lane d, λ HBV DNA + *Eco*RI + *Bgl*II; lane e, λ HBV DNA + *Eco*RI + *Hinc*II; lane f, λ HBV DNA + *Eco*RI + *Hind*III. The position of restriction fragments of plasmid pBR322 run on a separate lane are shown on the left.

*Permanent address: Hadassah University Hospital, Jerusalem, Israel.

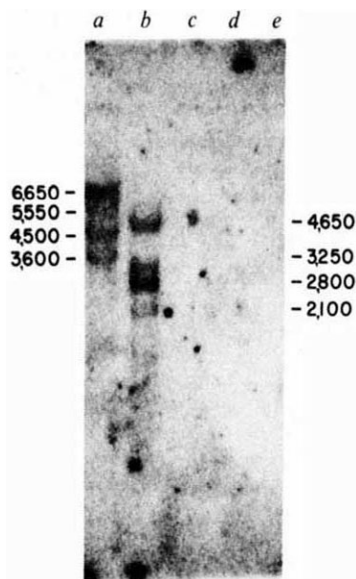


Fig. 2 Restriction enzyme analysis of total DNA from PLC/PRF/5 cells. PLC/PRF/5 cells were grown to confluence in monolayer culture using Eagle's minimal essential medium supplemented with glutamine, non-essential amino acids, 10% heat-activated fetal calf serum, penicillin, streptomycin and Fungizone. Trypsinized cells were lysed with detergent according to the procedure of Innis and Miller³³, nuclei were pelleted by centrifugation at 5,000g for 5 min, and DNA and nuclear RNA were obtained by SDS-phenol extraction followed by ethanol precipitation. Nuclear DNA was purified further by ribonuclease A digestion, followed by SDS-proteinase K treatment and phenol/chloroform/isoamyl alcohol extraction. DNAs from other tissues were purified essentially as described above, except that the starting material was total nucleic acids. Aliquots of 20 µg of each DNA sample were digested with *EcoRI* or *HindIII* in appropriate digestion buffer. To ensure complete digestion, 20 units of restriction endonuclease were used and incubation was at 37 °C. After 12 h of incubation, 20 additional units of fresh enzyme were added and incubation continued for an additional 12 h. Electrophoresis of these samples, their subsequent transfer to nitrocellulose paper and hybridization to ³²P-labelled pA01 HBV DNA were as described in Fig. 1 legend. The autoradiograph was developed by exposing the nitrocellulose paper for 5–7 days on Kodak XR-5 film using intensifying screens (Picker Corporation). Lane *a*, *HindIII*-digested PLC/PRF/5 DNA; lane *b*, *EcoRI*-digested PLC/PRF/5 DNA; lane *c*, *EcoRI*-digested rat liver DNA; lane *d*, *EcoRI*-digested normal human liver DNA; lane *e*, *EcoRI*-digested salmon sperm DNA. The length (in base pairs) of specific labelled fragments was determined by comparison with restriction enzyme fragments of plasmid pBR322 used as standards.

³²P-labelled HBV DNA as hybridization probe (specific activity 2×10^8 c.p.m. per µg). Lane *a* shows four HBV restriction fragments with *HindIII* of sequence length ~6,650, 5,550, 4,500 and 3,600 base pairs, respectively. The 5,550-base pair fragment reproduces poorly on this photograph but is present on all autoradiographs of this material. Lane *b* shows four HBV restriction bands with *EcoRI*, ranging from 2,100–2,200 to 4,650 base pairs. *EcoRI*-digested rat liver DNA (lane *c*), normal human liver DNA (lane *d*) and salmon sperm DNA (lane *e*) show no bands hybridizing with ³²P-HBV DNA. As one fragment with *EcoRI* and four fragments with *HindIII* are larger than cloned full-length double-stranded Dane particle DNA, these results suggest that HBV DNA sequences are integrated into PLC/PRF/5 cell line DNA. The possibility cannot be excluded that host or other virus-related sequences are associated with extrachromosomal HBV DNA to produce some of these bands. However, as *HindIII* recognizes no internal cleavage site in HBV DNA, the presence of four large fragments with this enzyme strongly favours integration of viral DNA sequences. The band migrating at 3,250 base pairs after *EcoRI* digestion (lane *b*) could represent a small amount of extrachromosomal (non-integrated) HBV DNA, and the 6,650-base pair band after *HindIII* treatment (lane *a*) could represent a dimer or circular concatamer of HBV DNA. Further studies will be necessary to characterize these bands fully.

The number and size of HBV restriction fragments suggest that more than one, but only a few, discrete host integration sites are involved. Although the lengths of fragments generated with *HindIII* are larger than those obtained with *EcoRI*, a more precise comparison cannot be made. This is especially true as HBV DNA integrated into different sites may contain deletions or rearrangements affecting the restriction pattern, and Dane particles from the original patient are not available for comparison.

To study viral RNA expression in this cell line, total cytoplasmic and nuclear RNAs were isolated and separated into poly(A)⁺ and poly(A)[−] fractions. RNA samples were applied to denaturing glyoxal slab gels, blotted to diazobenzylxymethyl (DBM)-cellulose paper and HBV sequences identified by hybridization with ³²P-HBV probe¹⁵. As shown in Fig. 3, total cytoplasmic poly(A)⁺ RNA (lanes *a* and *a'*) reveals two bands containing virus sequences, 21.5S (3,050 nucleotides) and 19.5S (2,500 nucleotides). Total nuclear RNA (lane *b*) shows three bands of 27S, 21.5S and 19.5S (the migration of all components in this fraction is retarded slightly by the presence of high molecular weight nuclear RNA). The 21.5S and 19.5S bands in nuclear RNA probably represent the same components as found in the cytoplasm. Cytoplasmic poly(A)[−] RNA (lane *d*) also shows a 27S band (4,700 nucleotides). Hybridisable material in the nuclear fraction is alkaline labile (lane *c*), ruling out the possibility of DNA contamination, and poly(A)⁺ RNA from normal human liver (lane *e*) shows no distinct radioactive bands, demonstrating the specificity of our HBV probe.

The biological activity and function of these viral RNAs are unknown. The presence of a poly(A) tail in the 21.5S and 19.5S RNA species suggests that they represent specific viral mRNAs. It has been reported that this cell line produces HBsAg^{1–3}, and a recent DNA sequencing study of Dane particle DNA proposes a

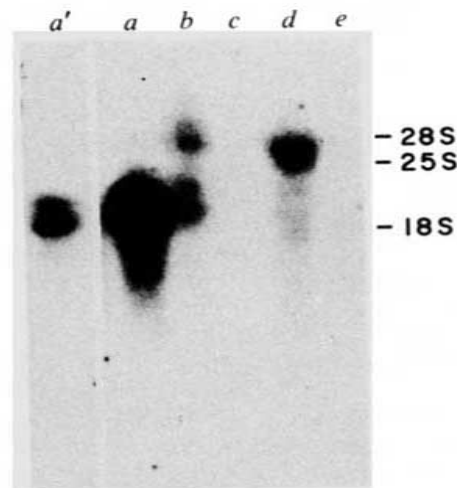


Fig. 3 Identification of RNA molecules containing HBV sequences in PLC/PRF/5 cells. Nuclei and cytoplasm from 2×10^8 PLC/PRF/5 cells were prepared as described in Fig. 2 legend. Isolated nuclei were resuspended and lysed in 50 mM Tris-HCl pH 7.5, 2 mM NaEDTA, 150 mM NaCl and 1% SDS. Nuclear RNA and DNA were isolated by phenol/chloroform/isoamyl alcohol extraction and nuclear RNA was recovered by repeated differential salt precipitation. Total cytoplasmic RNA was isolated from the post-nuclear supernatant fraction by SDS/phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation. Total cytoplasmic RNA was fractionated further into poly(A)⁺ and poly(A)[−] RNA by oligo(dT)-cellulose column chromatography. 15–60 µg of RNA was denatured by a glyoxal procedure³⁴ and electrophoresed on an agarose slab gel in Peacock-Dingman buffer³¹ for 5–6 h at 50 V. RNA was blotted to DBM paper¹⁶ and hybridized to nick-translated ³²P-labelled pA01 HBV DNA. After hybridization, the DBM paper was exposed on Kodak XR-5 film for autoradiography using intensifying screens. Lanes *a*–*e* were exposed for 3 days. Lane *a'* is lane *a* of the same gel exposed to autoradiography for only 17 h. Lane *a*, 15 µg of cytoplasmic poly(A)⁺ RNA; lane *b*, 50 µg of total nuclear RNA; lane *c*, 50 µg of alkali-treated total nuclear RNA; lane *d*, 60 µg of cytoplasmic poly(A)[−] RNA; lane *e*, 20 µg of cytoplasmic poly(A)⁺ RNA from normal human liver. The position of 18S, 25S and 28S ribosomal RNA markers determined in separate lanes is shown to the right.

892-base pair region as coding for this protein⁹; it is therefore surprising to find no viral RNA in the cytoplasm close to 1,000 nucleotides. Most probably, either the 21.5S or the 19.5S poly(A⁺) viral RNA codes for HBsAg. The finished polypeptide (molecular weight 25,000) may be derived by post-translational processing of a larger precursor. As the PLC/PRF/5 cell line produces only one known viral protein (HBsAg), the role of the other two viral specific RNAs remains unclear. These RNAs may code for nonstructural proteins which do not become incorporated into the mature virion, as observed in other systems after viral infection¹⁶.

At present, the major evidence linking HBV to hepatocellular carcinoma is epidemiological¹⁷. A variety of virus-cell interactions have been described in oncogenic transformation and/or persistent viral infection in cultured cells, so the PLC/PRF/5 line provides an excellent model for studying these phenomena as related to HBV gene expression. The ability of these cells to grow rapidly in tissue culture as well as in soft agar¹⁸, and to produce tumours in athymic *nu/nu* mice¹⁹ (our unpublished observations), indicates a malignant phenotype. However, in considering the relationship between the presence of viral genes and tumorigenicity, it is important to remember that transformation may result from altered cellular gene expression rather than the function of HBV genes. Therefore, the mere presence of integrated HBV genes with limited expression of HBV proteins does not prove viral oncogenicity, although these findings are consistent with observations in other systems¹⁹⁻²³. In spite of this limitation, evaluation of HBV genes and their expression in PLC/PRF/5 cells provides the first opportunity of studying the biology and pathobiology of HBV infection at the molecular level.

In the present study, viral DNA is transcribed into three specific RNAs. Although the function of these RNAs has not been determined, two are poly(A)⁺ (21.5S and 19.5S) and presumably represent cytoplasmic viral mRNAs. The third viral RNA is poly(A)⁻, is found in both the nucleus and the cytoplasm, and is larger than full-length Dane particle DNA. This suggests that the 27S RNA is derived by co-transcription of host and viral sequences from an integrated viral transcription unit, or that the full genome of hepatitis B virus in the cell is larger than that observed in Dane particles. Although other explanations are possible, evidence suggesting synthesis of small amounts of rapidly sedimenting RNA, containing viral and host sequences in molecules of larger apparent size than the viral genome, has been reported during integration of SV40 viral DNA²⁴⁻²⁸. In the present case, this material has been identified as a specific RNA molecule (27S or 4,700 nucleotides on agarose gel electrophoresis in strongly denaturing conditions) and represents a significant portion of viral sequences in both the nucleus and cytoplasm. The exact nature and role of 27S HBV RNA, as well as the other viral RNAs, remain to be determined.

After this manuscript was submitted, Marion *et al.*²⁸ reported similar findings of integrated HBV-DNA in the PLC/PRF/5 cell line.

We thank Dr J. Summers³⁰ for providing recombinant λ phage pA01 HBV DNA, Dr I. Millman for PLC/PRF/5 cells and Dr Probir Sarkar for advice. Research was supported in part by NIH grant AM 17702, American Cancer Society Institutional grant IN-28U and the Sara Chait Memorial Foundation. D.S. is the recipient of an NIH John E. Fogarty International Fellowship. These studies represent part of a thesis by N. R.-O. to be submitted to the Sue Golding Graduate Division, Albert Einstein College of Medicine, in partial fulfillment of the requirements for the degree of D.Phil.

Received 27 February; accepted 27 May 1980.

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Presence of integrated hepatitis B virus DNA sequences in cellular DNA of human hepatocellular carcinoma

Christian Brechot[‡], Christine Pourcel^{*}, Anne Louise^{*}, Bernadette Rain[†] & Pierre Tiollais^{*}

^{*} Unité de Recombinaison et Expression Génétique (INSERM U.163), Institut Pasteur, 28 rue du Dr Roux-75015 Paris, France

[†] Laboratoire d'Anatomopathologie (Service du Pr Ette.M), CHU Treichville-Abidjan, Côte d'Ivoire

Hepatitis B virus (HBV) may be one of the agents involved in the aetiology of human primary liver cancer¹. This hypothesis is supported by (1) the similarity between the geographical distribution of chronic carriers of the viral surface antigen (HBsAg) and that of hepatocellular carcinoma (HCC); (2) the increase in the prevalence of HBV markers in serum of patients with primary liver cancer when compared with the general population¹; (3) the observation that HBV infection precedes the development of the tumour¹. Moreover, these epidemiological indications of an association between HBV infection and hepatocellular carcinoma are supported by the detection of HBV markers such as HBsAg^{2,3} or viral DNA sequences, although in a non-integrated form^{4,5} in tumour tissue. To study the relationship between HBV and primary liver cancer further, we looked for the presence of free or integrated viral DNA in tumour tissue of human hepatocellular carcinomas and in a HBsAg-producing human hepatoma cell line⁶. Using the blot-transfer hybridization technique and cloned HBV DNA as a probe, we have now demonstrated that the viral DNA is integrated in the cellular genome both in tumour tissue and in a hepatoma cell line.

Tumoral tissue was an autopsy sample obtained from a 53 yr old man who lived in the savanna in the Ivory Coast (Ethnie Bete). HBsAg (subtype undetectable) and Anti-HBc were present in the serum (as tested by radioimmunoassay: AusRIA-II and Corab, Abbott Laboratories). HBV e

[‡] Present address: Unité de Recherches de Physiopathologie Hépatique (INSERM U.24), Hôpital Beaujon, Clichy, France.

antigen (tested by immunodiffusion) and anti-HBsAg (tested by radioimmunoassay: Ausab-Abbott Laboratories) were absent from the serum. Immunofluorescence, using anti-HBsAg and anti HBcAg labelled with fluoresceine isothiocyanate⁷, was negative in the tumour tissue. Normal liver tissue from this patient was not available.

The cellular DNA was extracted as described in Fig. 1 legend. Non-digested DNA and restriction fragments were analysed by agarose gel electrophoresis (Fig. 1a). We chose the restriction endonucleases *Eco*RI and *Hind*III because *Eco*RI cleaves the HBV genome once and *Hind*III does not cut any of the HBV genomes so far cloned^{8,9}. Cloned P-labelled HBV DNA was used as a probe to detect viral sequences⁸. As shown in Fig. 1a, hybridization of non-digested cellular DNA with the HBV probe occurred only in the high molecular weight (MW) DNA. Hybridization of the *Hind*III restriction fragments revealed the existence of five bands (Fig. 1a). Three bands corresponded to DNA fragments which migrated more slowly than the HBV genome and had MWs of 25,000, 19,000 and 12,000 respectively. Two bands of weaker intensity were located at the position of DNA fragments of MWs 3,200 and 2,400. Hybridization of the *Eco*RI restriction fragments revealed the presence of several bands (Fig. 1a). Three bands corresponded to DNA fragments of respective MWs 14,000, 8,000 and 6,500. Another particularly intense band was located at the position of the HBV genome. A similar pattern was observed with DNA extracted from tumour tissue of two other patients (Ivory Coast) with hepatocellular carcinoma (data not shown). No hybridization signal was observed with DNA extracted from a normal liver as a control (data not shown).

We analysed in the same way DNA from the hepatoma cell line PLC/PRF/5 (a generous gift from Dr J. Alexander) which was derived from a human hepatocellular carcinoma and which produced HBsAg (subtype ad)¹⁰. At the 100th passage, HBsAg was produced in the supernatant at a concentration of 50 ng ml⁻¹. Hybridization of non-digested cellular DNA occurred only in high molecular weight DNA (Fig. 1b). Analysis of *Hind*III restriction fragments revealed the presence of five bands of MWs 24,000, 17,000, 12,000, 6,500 and 4,200, respectively. Analysis of *Eco*RI restriction fragments revealed the presence of eight bands; one of these was intense and corresponded to a DNA fragment of MW 3,000 (Fig. 1b). The DNA pattern was identical at the 100th and 140th passages.

Taken together, these results demonstrate the integration of HBV DNA sequences in the cellular DNA of both the tumour tissue and the cell line. Our results differ from those of another study of human tumour tissue in which only free viral DNA was detected^{4,5}. However, our results obtained with the cell line PLC/PRF/5 are in agreement with those observed recently by Marion *et al.*¹¹, although the number and size of the restriction DNA fragments are different. The presence of several sharply delineated bands in the *Hind*III pattern of the tumour tissue (Fig. 1a) suggests the existence of a limited number of integration sites in the cellular DNA. These results are consistent either with the development of the liver tumour from one clone with several integration sites or with its development from a few clones each having particular integration sites. The difference in intensity of the bands could reflect a variability of the number of viral genomes or of the viral DNA length in the different integration sites. The very intense band located at the same position as that of HBV genome in the *Eco*RI pattern of the tumour tissue is not present in the non-digested DNA pattern. This result is consistent with the presence of two or more whole viral genomes inserted in tandem head-to-tail. The presence of a very small quantity of free viral DNA cannot be ruled out by our experiments.

The presence of integrated HBV DNA sequences in both the human hepatocellular carcinomas and in the cell line reinforce the interest of these two results. Indeed, the Alexander cell line produces HBsAg, possesses characteristics of malignancy⁶ and induces tumours in mice that are similar to human hepatoma¹². Demonstration of the integration of HBV genome in cellular

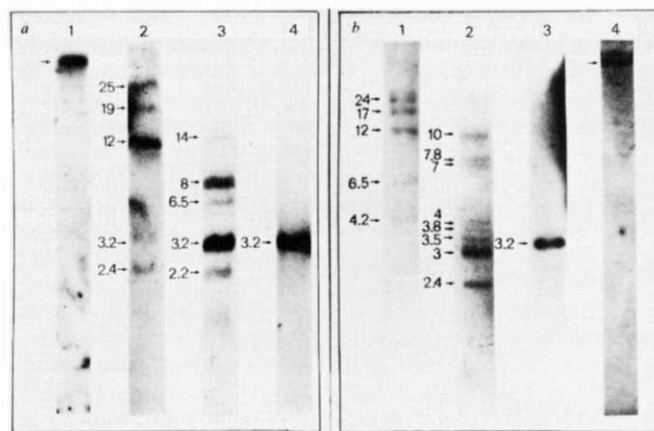


Fig. 1 The liver tumorous tissue is a postmortem sample obtained 6 h after death and kept frozen in liquid N₂. Tissue was pulverised in liquid N₂, then transferred into lysis buffer (Tris-HCl 10 mM, pH 7.8, NaCl 10 mM, SDS 0.5%, EDTA 10 mM) and digested with 500 µg ml⁻¹ of Pronase (Boehringer) at 37 °C for 2 h. DNA was extracted with phenol/chloroform, dialysed, treated with 100 µg ml⁻¹ of RNase A (Boehringer) and dialysed. Cellular DNA of the cell line was extracted by the same procedure using the same lysis buffer. The extracted DNA had an average MW of 100 × 10⁶. Digestion with restriction endonucleases *Eco*RI (Biolabs) and *Hind*III (Biolabs) was carried out at 37 °C for 8 h using 2 units of the enzyme for 1 µg of DNA and the appropriate reaction mixtures. Digestion products were precipitated with ethanol, dissolved in electrophoresis buffer (Tris-HCl, pH 7.8, 40 mM, sodium acetate 5 mM, EDTA 10 mM) and 0.2% agarose beads¹⁴. Electrophoresis was carried out in 0.8% agarose gels for restricted DNA and 0.4% gels for non-digested DNA using horizontal slabs of 20 cm long and 0.5 cm thick at 1 V cm⁻¹ for 24 h. DNA was then transferred to nitrocellulose filter by a modification of the Southern technique described by Wahl *et al.*¹⁵. The filter was hybridized using cloned HBV DNA⁸ as a probe and the nick-translation procedure¹⁶. [α -³²P]dCTP (Amersham, 2,000 Ci mmol⁻¹) and [α -³²P]dTTP (Amersham, 2,000 Ci mmol⁻¹) were used and the probe yielded a specific activity of 4 × 10⁸ c.p.m. per µg. The hybridization procedure used dextran sulphate as described by Wahl *et al.*¹⁵; 5 × 10⁶ c.p.m. of probe was added to each filter. The nitrocellulose filter was autoradiographed with preflashed Kodak X-Omat R film in the presence of a Dupont Lightning Plus screen. **a**, Tumour tissue: lane 1, 30 µg of non-digested cellular DNA; lane 2, 80 µg of cellular DNA digested with *Hind*III; lane 3, 80 µg of cellular DNA digested with *Eco*RI; lane 4, 30 µg of cellular DNA from normal liver cells mixed with 10 pg of cloned HBV DNA. **b**, Cell line PLC/PRF/5: lane 1, 20 µg of cellular DNA digested with *Hind*III; lane 2, 20 µg of cellular DNA digested with *Eco*RI; lane 3, 10 pg of cloned HBV DNA; lane 4, 20 µg of non-digested cellular DNA. Cloned HBV DNA digested with *Bam*HI and λ plac5cl857S7 DNA digested with *Hind*III were used as molecular weight markers.

DNA is an additional argument for HBV as a factor in human hepatocellular carcinoma. Until now, integration of viral DNA in the genome of human tumours has only been confirmed for Epstein-Barr virus in Burkitt's lymphoma and some nasopharyngeal carcinomas¹³. Our observation is another example of such a relationship between a DNA virus and human cancer. However, the demonstration of integrated HBV DNA sequences is not sufficient to assert the role of HBV in the onset of human primary liver cancer. Additional studies should compare the pattern observed in non-tumorous and tumorous liver cells.

We thank Professors Attia, Beda, Loubienes and Drs Carsuzaa, Condat and Soubeyran for providing autopsy samples, Professor Benhamou and Mr Roland Nageotte for helpful discussion, and Miss Michelle Fonck for technical assistance.

Received 24 March; accepted 5 June 1980.

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Integration of hepatitis B virus sequences and their expression in a human hepatoma cell

Jeffrey C. Edman, Patrick Gray, Pablo Valenzuela, Leslie B. Rall & William J. Rutter

Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143

Hepatitis derived from hepatitis B virus (HBV) infection is endemic throughout the world, but it is particularly prevalent in Asia and Africa¹. In these areas, demographic studies show a strong coincidence between HBV infection (assayed by HBV antigenic markers) and the incidence of primary liver cancer. On these grounds, a causal link between HBV infection and primary hepatocellular cancer has been proposed^{2–5}. Recently, a human hepatoma cell line (PLC/PRF/5; Alexander cells) has been shown to produce hepatitis B surface antigen (HBsAg)⁶. We show here that the Alexander cell line contains at least six (four complete and two partial) hepatitis B viral genomes integrated into high molecular weight host DNA. An analysis using specific probes to fragments of the HBV genome suggests that integration of the virus in most cases occurs at the nicked cohesive end region of the virus. Expression of viral sequences using Northern blots demonstrates the presence of RNA transcripts specific for the surface antigen sequences of HBV DNA and the absence of detectable transcripts corresponding to the hepatitis B core antigen.

The study of the possible role of HBV in cell transformation has been hampered by the lack of an *in vitro* system for propagation of the virus. However, with the Alexander cell, which is derived from a hepatoma obtained from a male individual from Mozambique, HBsAg accumulates in the media as characteristic 22-nm particles^{6,7}. The HBsAg of Alexander cells is identical to that isolated from HBV carriers and contains the unglycosylated and glycosylated forms of HBsAg protein (molecular weights (MWs) 25,000 and 29,000, respectively)^{8,9}. In contrast, the Alexander cells do not produce hepatitis B core antigen (HBcAg; MW 19,000⁸), the cryptic HBcAg¹⁰ or free virus (Dane particles)¹¹. The presence of the endogenous Dane particle DNA polymerase that fills in the partially single-stranded region of HBV DNA to generate a double-stranded circular structure with cohesive ends¹² has not been tested. These cells show typical epithelial cell growth and exhibit many of the functions of liver cells. They also induce HBsAg-positive tumours in nude mice¹³.

We have examined the physical state and expression of the HBV sequences in Alexander cells using the techniques developed by Southern for DNA analysis¹⁴, 'Northern' blots for RNA analysis¹⁵ and nick-translated cloned HBV sequences as probes¹⁶. Figure 1 shows the hybridization with the cloned HBV DNA probe to *Hind*III, *Eco*RI and *Hind*III-*Eco*RI digestions of high molecular weight DNA prepared from Alexander cells. The autoradiograph shows the presence of several large DNA fragments that hybridize with the probe. *Hind*III digestion (lane 2) yields at least six hybridizing bands, ranging from 4.2 to >30

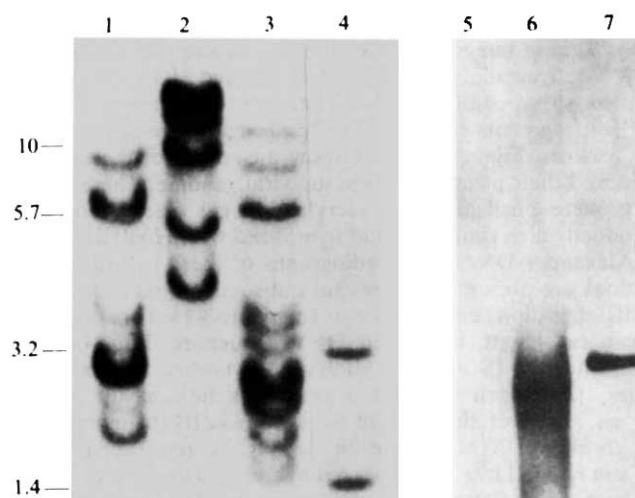


Fig. 1 Hybridization of ³²P-labelled HBV DNA probe to *Eco*RI (lane 1), *Hind*III (lane 2) and *Hind*III-*Eco*RI (lane 3) digests of Alexander cell DNA; standards were derived from cloned HBV DNA (lane 4, 3,200 and 1,400 base pairs), *Eco*RI-digested normal human liver DNA (lane 5), *Eco*RI-digested chronic hepatitis liver DNA (lane 6) and Dane particle DNA made double stranded by endogenous DNA polymerase reaction (lane 7). DNA was prepared from Alexander cells by suspending 0.5 ml of packed cells in 5 ml of 10 mM Tris-HCl (pH 7.4), 10 mM NaCl and 2 mM EDTA-Na₂. SDS and proteinase K were then added slowly to 2.5% and 0.1%, respectively. The solution was incubated for 18–24 h at 37 °C, extracted once with phenol, once with chloroform/isoamyl alcohol (24:1) and precipitated with two volumes of ethanol. The precipitate was resuspended in 5 ml of the same buffer, RNase A and RNase T1 added to 40 µg ml⁻¹ and 10 µg ml⁻¹, respectively, and digested for 1 h at 37 °C. The phenol and chloroform/isoamyl alcohol extractions were repeated, and the DNA precipitated with ethanol and resuspended in 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA. DNA from liver samples was prepared in an identical manner except the tissue was homogenized briefly with a Teflon homogenizer before the addition of SDS and proteinase K. Dane DNA was made completely double stranded and extracted according to Landers *et al.*¹². Restriction enzyme (New England Biolabs) digests were carried out according to the manufacturer's instructions. Completion of digests was monitored by addition of 1 µg of λ DNA to a small fraction of the digest and digested DNA was precipitated with ethanol. The samples were run on 1% agarose gels. DNA was transferred to nitrocellulose filters according to Southern¹⁴ and hybridized to nick-translated²³ HBV probe in the presence of 10% dextran sulphate²⁴. Numbers on the left-hand side indicate the fragment sizes in kilobase pairs.

kilobases. The unit length in all six is greater than that of the HBV DNA (3.2 kilobases). As there are no *Hind*III sites in any of the cloned HBV sequences reported, the data suggest at least six different sites for the integration of HBV DNA into host cell DNA. There is no detectable free viral DNA at 3.2 kilobases; this is consistent with the lack of virus production of these cells. This pattern of hybridization is observed in Alexander cells after many passages in tissue culture and also in recloned populations of the cells. Thus, the multiplicity of bands does not reflect cell heterogeneity.

*Eco*RI digestion (Fig. 1, lane 1) produces a series of 10 bands ranging from 2.4 to 9.5 kilobases with a particularly intense band at 3.0 kilobases. As HBV DNA contains a single *Eco*RI site, it is expected that *Eco*RI digestion would give twice as many hybridizing fragments as *Hind*III-restricted DNA. Ten rather than twelve bands were observed. This discrepancy may be the result of overlap of bands at the 3.0 kilobase band, as an analysis using a more highly resolving gel system shows that there are multiple bands in this region (data not shown). Figure 1 also shows the results of a similar analysis of HBV viral DNA (lane 7), normal human liver DNA (lane 5) and DNA from the liver of a chronic hepatitis patient (lane 6). No hybridization signal was detected in the DNA from normal liver; thus, there are no sequences closely related to HBV DNA in the normal human genome. Hybridization characteristic of free viral DNA was detected from the DNA of a chronic hepatitis liver.

To determine the fraction of HBV information retained in each of the six integrated viral sites, we hybridized Alexander DNA with fragments of HBV DNA. Cloned HBV DNA was digested with a combination of restriction enzymes to yield a series of fragments each representing roughly one-sixth of the viral genome. Figure 2a and b show the source of these fragments and their positions within the viral genome. These fragments were purified by polyacrylamide gel electrophoresis, individually nick translated and hybridized with *Hind*III-digested Alexander DNA. Autoradiographs of these hybridization reactions are presented in Fig. 3a and summarized in Fig. 2c. *Hind*III digestion generates the six fragments which presumably contain full-length integrated HBV sequences. Four of the bands (30, 27, 18 and 5.7 kilobases) hybridize with all the probes; thus, each contains a major fraction of the HBV genome. However, the 10-kilobase band *Hind*III fragment does not hybridize with the *Bgl* 3 probe (see Fig. 2 legend) and the 4.2 kilobase *Hind*III fragment does not hybridize with the *Bgl* 3 and *Bgl* 2 probes and therefore contain partial HBV genomes. The absent sequences of both partial genomes are in the region of the core antigen gene. The apparent deletion of HBV information in the 10- and 4.2-kilobase fragments could also be explained by the presence of additional *Hind*III sites in these two integrated copies. This would generate an additional *Hind*III fragment

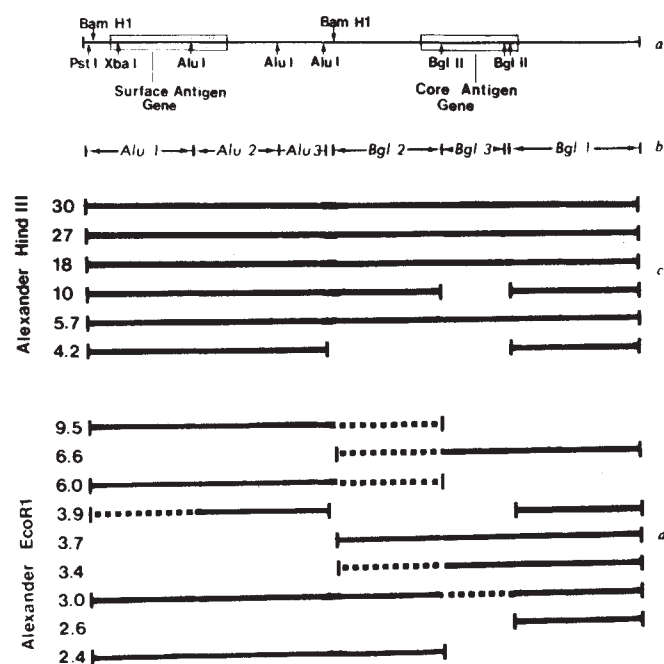


Fig. 2 Source of HBV DNA fragment probes and summary of hybridization to Alexander cell DNA digests. *a*, Restriction map of cloned HBV DNA linearized by *Eco*RI¹⁶; *b*, fragment probes used in the hybridization reactions. Cloned HBV DNA was digested with *Bam*HI and *Eco*RI, and the 1,800- and 1,400-base pair fragments purified by agarose gel electrophoresis and electroelution. The 1,800-base pair fragment was digested with *Bgl*II to yield fragments *Bgl* 1–*Bgl* 3 and the 1,400-base pair fragment with *Alu*I to yield *Alu* 1–*Alu* 3. Fragments derived from these digests were purified by polyacrylamide gel electrophoresis and individually nick translated. The sizes of the various fragments (in base pairs) are: *Alu* 1, 714; *Alu* 2, 344; *Alu* 3, 242; *Bgl* 1, 789; *Bgl* 2, 584; *Bgl* 3, 421. *c*, Summary of hybridization of fragment probes to *Hind*III-digested Alexander cell DNA. The sizes (in base pairs) of *Hind*III fragments hybridizing to the entire HBV probe are given on the left. A solid bar represents a positive hybridization result and absence of a bar represents no hybridization to the same regions as shown in *a* and *b*. For example, the 10-kilobase *Hind*III fragment of Alexander cell DNA does not hybridize to the *Bgl* 3 probe. *d*, Summary of hybridization of fragment probes to *Eco*RI-digested Alexander cell DNA. As in *c*, *Eco*RI fragment size is given on the left. A solid bar represents strong hybridization, a dashed bar weak hybridization. Two patterns of hybridization are evident. The 9.5-, 6.0-, and 2.4-kilobase bands hybridize from the *Eco*RI site through the surface antigen gene with the *Bgl*2 region and the 6.6-, 3.7- and 3.4-kilobase bands hybridize from the *Eco*RI site through the core gene into *Bgl*2. Anomalous hybridization is observed in the 3.9-, 3.0- and 2.4-kilobase bands.

hybridizing to the apparently deleted regions. No such fragments were detected.

The orientation of HBV DNA sequences within the Alexander genomic DNA has been studied by hybridizing the specific probes to *Eco*RI-digested Alexander DNA (Fig. 3b). As there is a single *Eco*RI site in the viral genome and assuming that integration is not random with respect to the virus, two patterns of hybridization should be observed, one hybridizing to fragments of HBV from the HBV *Eco*RI site clockwise to the region of integration, and the other pattern going counterclockwise. As summarized in Fig. 2d, two such patterns are obtained. One hybridizes from the *Eco*RI site through the surface antigen gene to a region approximately 1,600 base pairs from the *Eco*RI site; the other hybridizes approximately 1,600 base pairs in the opposite direction. Thus, *Eco*RI cleavage essentially bisects the integrated genome. This places the integration site in the region of the cohesive end region of Dane DNA¹⁷. There are three exceptions to this general pattern. One is the 3.9 kilobase fragment that hybridizes to both sides of the *Eco*RI site in one of the integrated regions. Another exception is the 2.6 kilobase band hybridizing only to a small region of HBV. This probably arises from the integration of a partial HBV genome. Finally, the 3.0 kilobase band apparently hybridizes with all regions of the genome. The hybridization intensity varies with different probes and is particularly weak with the *Bgl* 3 probe. The width of the band also varies, being nearly resolved into two bands using *Alu* 1 as a probe and appearing as a single band using *Bgl* 1. As this band may be resolved into three bands, it is possible that the anomalous hybridization in this region is due to a multiplicity of bands representing a combination of fragments having the two hybridization patterns. Other possible explanations are tandem integration of the virus or the loss of an *Eco*RI site in one of the viral copies coupled with the presence of *Eco*RI sites flanking the viral sequences.

The orientation of HBV integration may be responsible for the selective pattern of HBV gene expression in these cells. An analysis of the expression of HBV was carried out by hybridization of HBV probes to poly (A)-enriched Alexander cell RNA fractionated into size classes by electrophoresis in denaturing conditions¹⁸. This RNA was transferred to diazotized paper as described by Alwine *et al.*¹⁸ and then hybridized with cloned HBV DNA probe (Fig. 4). The predominant hybridization signal is a high molecular weight RNA approximately the size of 18S ribosomal RNA (~2,000 bases). This band hybridizes specifically to the *Alu* 1 fragment (see Fig. 2 legend) of the HBV genome that contains the surface antigen gene but includes 300 base pairs 5' to the gene (the *Bgl* A probe, see Fig. 4 legend) and 700 base pairs 3' to the gene (the *Bgl* B probe). This major transcript does not hybridize to the *Bgl* 1, *Bgl* 2 or *Bgl* 3 probes, demonstrating the absence of core antigen-specific transcripts. These studies suggest that at least 1,500 base pairs of the HBV genome hybridize to the transcript. As this is smaller than the total transcript, additional sequences may be contributed by transcription of sequences of the Alexander genomic DNA and/or by poly (A) addition. HBV probes also hybridized to larger and smaller RNA sequences than the major transcript. This hybridization is HBV-specific as negligible background is observed in the hybridization of HBV DNA to uninfected liver RNA (data not shown). The HBV-related RNA which is larger than the major transcript is obviously of considerable significance because it must represent either a precursor to the approximately 2,000-base major transcript or an independent transcript(s). The lower molecular weight material probably represents processing or degradation of the major transcript, as is seen in other systems.

Others have previously investigated the question of HBV integration into the host cell genome. Summers *et al.*¹⁹ studied a series of six hepatomas using nick-translated HBV DNA. They concluded that some of the tumours contained HBV DNA but that none contained integrated virus. Marion *et al.*²⁰ have recently reported the integration of HBV in Alexander cells. They detected only three *Hind*III fragments hybridizing to total

HBV probe, roughly corresponding to the lower three bands found in Fig. 1, lane 2. The source of this discrepancy is uncertain; however, it is not a result of partial cleavage by restriction enzymes in our experiments. The present experiments demonstrate that HBV sequences are integrated into a minimum of six different sites in Alexander cell DNA. The results suggest that the nicked cohesive end region of HBV DNA coincides with the region of integration of at least several of the HBV fragments. A circular structure with terminally redundant ends has been suggested as the precursor to integration in retroviruses, known integrating viruses^{21,22}. Such a structure could be formed in HBV by repair of the nicked region with the endogenous polymerase activity, resulting in a structure that contains a terminally redundant sequence.

Integration of HBV in a malignant cell does not imply that the virus is the oncogenic agent. However, viral transformation is usually associated with the integration of viral sequences into host chromosomal DNA. If HBV is an oncogenic virus, the transformation event producing the Alexander cell might have involved the gene product HBsAg or be some function of the integration of HBV. Analysis of the integration of HBV in other

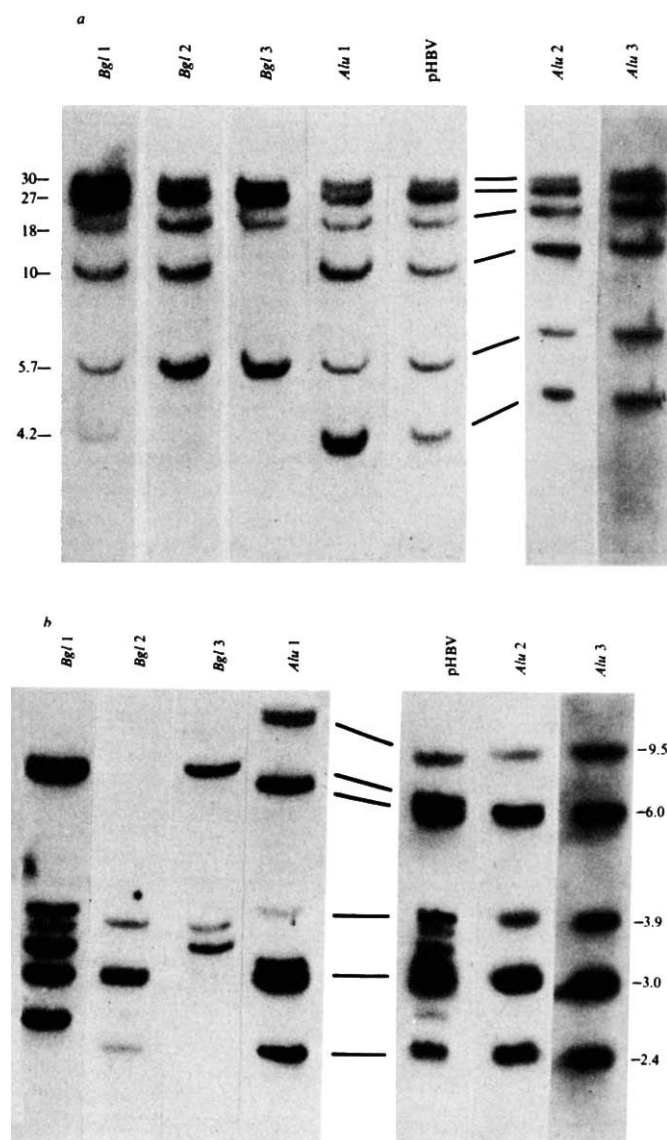


Fig. 3 Hybridization of *Hind*III (a) and *Eco*RI (b) digests of Alexander DNA with probes representing fragments of HBV DNA. Digestion products were run on 0.8% agarose gels and blotted to nitrocellulose filters. Individual lanes were cut out and hybridized to probes derived from cloned HBV DNA. The various probes are described in Fig. 2a,b. pHBV represents probe prepared from the entire HBV sequence. These hybridizations are summarized in Fig. 2c,d. Fragment sizes are indicated in kilobase pairs.

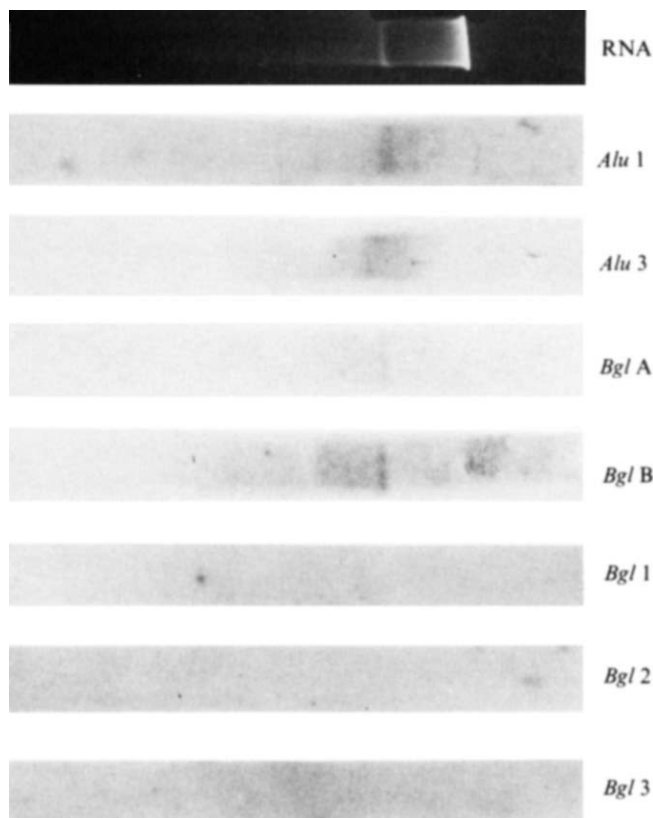


Fig. 4 Hybridization of poly(A)-enriched Alexander cell RNA with ³²P-cloned HBV DNA fragments. RNA was isolated from Alexander cells by the method of Chirgwin *et al.*²⁵ and passed once over oligo(dT)-cellulose. This RNA was run on a methyl mercury agarose gel and transferred to diazotized paper as described by Alwine *et al.*¹⁵. Individual lanes were cut out and hybridized to the various probes as shown in Fig. 2b. In addition, two other probes were used: *Bgl* A was derived from *Bgl* 1 and represents a 100-base pair fragment adjacent to the *Eco*RI site, and *Bgl* B is derived from *Bgl* 2, being 100-base pairs from the *Bam*HI site. RNA lane shows an ethidium bromide stain of the gel before transfer.

hepatomas, as well as efforts to obtain the expression of the HBsAg gene in mammalian cells, may help to clarify this issue. However, the putative role of HBV in transformation itself will be greatly facilitated by the development of an *in vitro* cell system that is capable of propagation and integration of the virus.

We thank Drs J. Birnbaum, M. Hilleman, R. Hirschman and A. Tytell for the initial supply of Alexander cells and Dane particle preparations, Dr E. Smuckler for human hepatic liver, Dr H. M. Goodman for helpful discussion, and L. Spector for preparation of the manuscript. P.G. is a fellow of the Damon Runyon-Walter Winchell Cancer Fund. This research was supported by a grant from Merck Sharpe & Dohme Research Laboratories.

Received 20 March; accepted 18 June 1980.

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Partial deletion of the α -globin structural gene in human α -thalassaemia

Stuart H. Orkin & Alan Michelson

Division of Hematology-Oncology, Children's Hospital Medical Center, Sidney Farber Cancer Institute, Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115

Defective synthesis of specific polypeptide chains of haemoglobins is the hallmark of human disorders known collectively as the thalassaemia syndromes¹. Genetic lesions intimately linked to or within the globin genes are responsible for these conditions. Recently, the molecular basis of several forms of thalassaemia has been clarified by examination of the organization and structure of globin genes. Gene mapping studies using the gel blotting technique of Southern² have revealed an array of deletions involving globin structural genes in thalassaemias^{3–12} and nucleotide sequencing has identified a point mutation responsible for thalassaemia in one individual¹³. In most α -thalassaemias, deletion of entire α -globin structural genes has been observed^{3,4}. In specific forms of β -thalassaemia in which δ -globin chain expression is affected ($\delta\beta$ -thalassaemia, $\gamma\delta\beta$ -thalassaemia and hereditary persistence of fetal haemoglobin syndrome), various deletions within the cluster of closely linked γ -, δ - and β -globin genes occur^{5–9}. In some Asian Indian individuals with β^0 -thalassaemia, an internal deletion in the DNA involving the 3'-portion of the β -globin gene has been described^{10–12}. Lastly, in one Chinese patient with β^0 -thalassaemia, a nonsense mutation within the β -sequence has been identified¹³. We now report a new genetic lesion present in some individuals with α -thalassaemia: an extensive deletion of the 5' portion of the α -globin structural gene. This defect has been characterized by DNA sequence analysis of cloned α -thalassaemia genes.

In normal humans the α -globin structural genes are duplicated and closely linked¹⁴. By gel blotting experiments these genes have been localized to a 22.5 kilobase fragment of genomic DNA generated by the restriction enzyme *EcoRI*. In α -thalassaemic DNA samples, several different alleles of the normal complex are observed. These include *EcoRI* fragments of 22.5, 18–20 and 2.6 kilobases in length^{15,16}. The 22.5-kilobase fragment, grossly normal by restriction mapping, contains two structural genes, one of which is presumed to be functionally abnormal on the basis of its presence in some individuals with HbH disease α -thalassaemia, a condition in which only one functional α -globin gene exists. The 18–20-kilobase fragment contains a single α -gene that most commonly arises due to non-homologous crossing-over within the region of the duplicated structural genes. We focus here on the 2.6-kilobase fragment, identified only in several individuals with HbH disease originating from the Mediterranean basin. On the basis of DNA–DNA hybridization^{15,16}, this fragment seemed to

contain at most a single α -globin structural sequence. To characterize this novel restriction fragment in detail, we have cloned it in bacteriophage λ vectors from two unrelated individuals with α -thalassaemia and analysed its structure by restriction mapping and DNA sequencing.

The α -specific 2.6-kilobase fragments were cloned from these two individuals by different strategies. The lymphocyte DNA of patient 1 (HbH disease genotype: 18–20 kilobases/2.6 kilobases¹⁵) was digested with *EcoRI* and fragments of 2.2–2.8 kilobases were isolated by preparative agarose gel electrophoresis. This enriched DNA fraction was ligated to the outer arms of phage λ gtWES (ref. 17) and packaged *in vitro* into viable phage¹⁸, and recombinants were screened by the plaque assay method of Benton and Davis¹⁹ using total ($\alpha + \beta$) ³²P-labelled globin cDNA as probe. Positive phages were then rescreened using nick-translated DNA from the plasmid α -cDNA clone JW101²⁰. Among approximately 3×10^5 recombinants, one α -globin-specific phage was isolated. Analysis of its DNA indicated that, in addition to a 2.6-kilobase fragment, at least two other DNA fragments in the 2–3-kilobase size range were present. The 2.6-kilobase fragment was recloned in λ Charon 16A (ref. 21) free of these other human fragments. This phage was designated $\lambda\alpha$ T1(2.6). A 'library' of the lymphocyte DNA of patient 2 (HbH disease genotype: 22.5 kilobases/2.6 kilobases) was prepared in λ Charon 4A (ref. 21) by the procedure of Kemp *et al.*²². Screening of this library constructed from partial *EcoRI** digestion fragments yielded several α -specific phage containing the 2.6-kilobase fragment. From one such isolate the 2.6-kilobase fragment was subcloned in λ Charon 16A and designated $\lambda\alpha$ T2(2.6).

Gel blotting of these recombinant phage with α -globin probes specific for sequences either 5' or 3' to the *HindIII* site normally present at the codons for amino acids 90/91 of the α -sequence^{14,23} demonstrated that coding sequences from both regions were present in the 2.6-kilobase *EcoRI* fragment (Fig. 1). Additional restriction mapping immediately raised questions regarding the nature of the cloned globin gene. For example, cleavage sites for *MboII* and *TaqI* located normally in the α -sequence within the 5'-untranslated region and at the codons for amino acids 46/47 (ref. 23), respectively, were not present in the anticipated positions 5' to the intragenic *HindIII* site (Fig. 2). *TaqI* and *MboII* sites located approximately 55 and 170 base pairs 3' to the *HindIII* site were located within what was found to be an intervening sequence of approximately 150 base pairs within the cloned α -thalassaemia gene (see below and Fig. 2). The presence of these latter restriction sites in this α -globin gene is in accord with recent findings regarding intervening sequences of cloned normal human α -genes²⁴. In agreement with our previous mapping of genomic DNA, *BamHI* and *HpaI*¹⁵, as well as *SacI* and *BglII*, did not cut the 2.6-kilobase fragment.

To determine the nature of the cloned globin gene and to localize the lesion responsible for thalassaemia in this instance, DNA sequencing was carried out using the chemical degradation method of Maxam and Gilbert²⁵. The sequencing strategy and a partial sequence of the gene from approximately –170 to +60 base pairs relative to the intragenic *HindIII* site are shown in Figs 2–4. The nucleotide sequence predicts the correct α -globin amino acid sequence from amino acids 57 to 99. An intervening sequence, the initial portion of which is shown, interrupts the coding sequence after codon 99, as is the case in the mouse α -globin gene²⁶ and probably also the normal human α -globin genes²⁴. There is an abrupt loss of the α -globin coding sequence 5' to codon 57 (Figs 3, 4). The DNA sequence of this region does not encode any globin-like polypeptide. In fact, it contains a termination codon (UGA) at the position expected for codon 48. Gel blotting experiments (not shown) did not reveal any hybridizable α -globin sequences 5' to the *HinfI* site depicted in Fig. 2, therefore indicating that the missing 5' portion of the α -sequence is not contained within the cloned 2.6-kilobase fragment. Additional DNA sequencing, to be reported elsewhere, has revealed a complete α -globin sequence for codons 100–141 following the intervening sequence as well

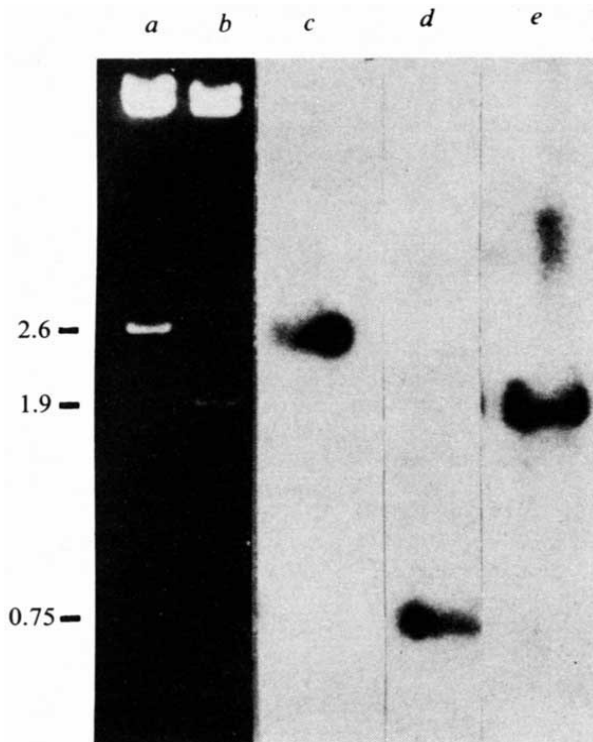


Fig. 1 Gel blotting of λ T1(2.6) DNA with 5'- and 3'-specific α -probes. Recombinant phage DNA (0.5 μ g) was digested with *Eco*RI alone (lanes a,c) or *Eco*RI+*Hind*III (lanes b, d, e) and electrophoresed in 1% agarose. Ethidium bromide (EtBr) staining of the DNA is shown in lanes a,b. Lanes c,d,e represent hybridization to 5'+3'-specific, 5'-specific and 3'-specific α -globin DNA probes, respectively. 5'+3'-specific probe was prepared by nick translation of plasmid JW101 DNA²⁰. Probes specific for α -sequences 5' and 3' to the intragenic *Hind*III site were prepared from JW101 DNA digested with *Hind*III, as described by Embury *et al.*⁴. The sizes of fragments resulting from digestion of the cloned segment are shown in kilobases. Gel blotting of λ T2(2.6) DNA yielded identical results.

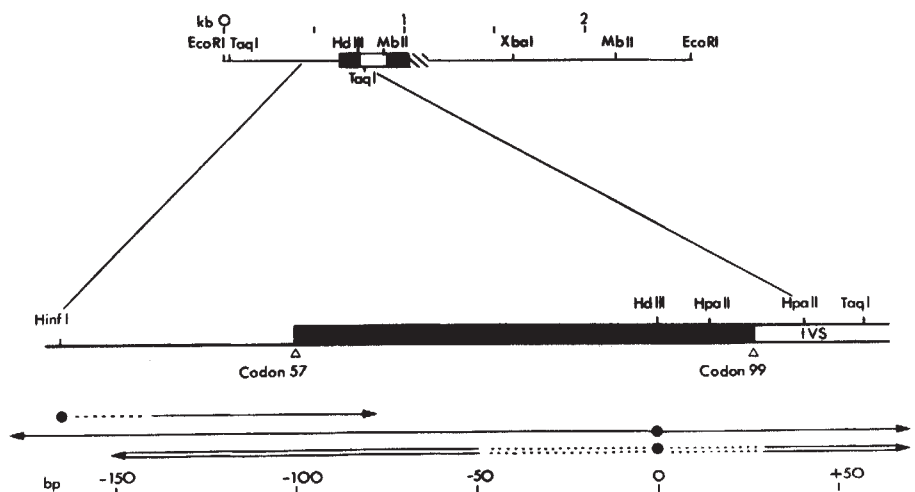
as the presence of a 3'-untranslated region exhibiting several scattered single nucleotide changes from the published normal mRNA sequence²³. Whether these changes reflect previously unrecognized sequence polymorphisms within α -globin genes or sequence divergence from normal in these α -thalassaemia genes is uncertain at present. Thus, the striking feature of the structure of the α -globin gene present in the 2.6-kilobase fragment is the absence of the 5'-untranslated region and codons 1-56.

Two observations show this structurally abnormal globin gene is not an artefact of cloning. First, the identical deletion has been observed in the fragment cloned from unrelated individuals by different cloning strategies. Furthermore, several independent recombinant phages with the same structure of the 2.6-kilobase fragment have been isolated from the library constructed from the DNA of patient 2. The precise reproducibility with which this fragment has been isolated argues strongly against its accidental creation during the cloning procedures. Second, in gel blotting experiments, the 2.6-kilobase *Eco*RI fragment of genomic DNA of these patients hybridizes in the presence of a vast excess of unlabelled JW101 DNA with probe prepared from the portion of the 2.6-kilobase fragment 5' to the intragenic *Hind*III site (not shown). Thus, sequences 5' to codon 57 in the cloned fragment reside in the same location in genomic DNA.

The 2.6-kilobase fragment, therefore, seems to have arisen by a deletion originating 5' to a normal α -globin gene. In theory, a large insertion of DNA within the coding segment of the gene might have occurred. In this case, the missing 5' portion of the α -gene would be located elsewhere in the genome and be represented in gel blotting experiments as an additional restriction fragment. As analysis of genomic DNA of individuals with the 2.6-kilobase *Eco*RI fragment has not revealed such additional hybridizing bands^{15,16}, a deletion rather than an insertion of DNA is the likely origin of this structurally abnormal globin gene. Note that this is the first partially deleted globin gene in which the deletion terminates within the coding region. In the partial deletion of δ -globin sequences in one form of $\delta\beta$ -thalassaemia⁶ and of β -sequences in β^0 -thalassaemia¹⁰⁻¹² the deletions end within the large intervening sequences. On the basis of its distance from the 3' *Eco*RI site^{14,15} and the presence of specific restriction sites in its 3'-flanking region (for example, extragenic *Xba*I site²⁷), the α -globin sequence of the 2.6-kilobase fragment is probably derived from the 3'- α -globin gene of the normal duplicated complex.

By gene cloning, Lauer *et al.*²⁴ have determined the arrangement of α -like globin genes in normal human DNA as

Fig. 2 Restriction map of the 2.6-kilobase cloned fragment and the strategy for DNA sequencing. The map of the cloned 2.6-kilobase fragment of λ T1(2.6) (upper portion) was constructed from the results of gel blotting and partial restriction mapping by the method of Smith and Birnstiel²⁸ after end-labelling of the *Hind*III site. The expanded segment below indicates the region of the cloned insert that was sequenced by the method of Maxam and Gilbert²⁵. The solid dots indicate the fragment ends that were labelled. Single arrows indicate fragments labelled at the 5' end with [³²P- γ]ATP by polynucleotide kinase²⁵. Double arrows indicate fragments that were 3' labelled with [³²P- α]dNTPs by the action of the Klenow fragment of *Escherichia coli* DNA polymerase. The solid portions of these arrows indicate those regions for which unequivocal sequences for each fragment were obtained. HdIII = *Hind*III; MbII = *Mbo*II; IVS = intervening sequence. The filled boxed regions indicate globin coding regions. The open boxed area represents the nearly 150-base pair intervening sequence present in the cloned gene. 3'-Untranslated sequences are shown by the cross-hatched area. The cloned fragment is orientated with its 5' end to the left.



Cloned Gene	TGGAATCTTAGCATTCTGGGAGGCCAAGTGGGATGGTCACTTGGAGCTGGAGTTCAAGACCACTCA
α -Globin	LeuSerPheProThrThrLysThrTyrPheProHisPheAspLeuSerHisGlySerAlaGlnValLysGlyHisGlyLysLysVal
α -mRNA	CUGUCCUCCCCACCAAGACCUACUCCCGGACUUGACCGACGCGCUCGCCAGGUUAGGGCCACGGCAAGAAGGUG
Cloned Gene	GCCGACGCGCTGACCAACGCCGTGGGCGACGTGGACGACATGCCAACGCGCTGTCCGCCCTGAGCGACCTGCACGCCACAAGCTT
α -Globin	AlaAspAlaLeuThrAsnAlaValAlaHisValAspAspMetProAsnAlaLeuSerAlaLeuSerAspLeuHisAlaHisLysLeu
α -Globin	GCCGACGCGCUGACCAACGCCGUGGCGCACGUGGACGACAUGCCAAACGCGUGUCCGCCUGAGCGACCGCCACAAGCTU
Cloned Gene	CGGGTGGACCGGTCAACTTCAAG-----Intervening Sequence-----
α -Globin	ArgValAspProValAsnPheLysLeuLeuSerHisCysLeuLeuValThrLeuAlaLa....
α -mRNA	CGGUGGACCGGCUCAACUCCAGCUCCUAGGCCACUGCCUGUGUGACCGUGGCCG....

Fig. 3 Partial nucleotide sequence of the 2.6-kilobase fragment. The nucleotide sequence of the portion of the cloned insert shown at the bottom of Fig. 2 is compared here with the amino acid and nucleotide sequences of normal α -globin and α -globin mRNA²³. The underlined segment 5' to the position of codon 57 does not resemble normal α - or other globin sequences. The nucleotide sequence of $\lambda\alpha$ T2(2.6) was identical in the region shown here.

5'- ζ 2- ζ 1- $\psi\alpha$ 1- α 2- α 1-3' where ζ 1 and ζ 2 are embryonic α -like genes, $\psi\alpha$ 1 a non-functional α -like gene, and α 1 and α 2 the normal adult α -genes. Restriction maps of the ζ and $\psi\alpha$ 1 genes and their flanking regions do not resemble the pattern of sites within our 2.6-kilobase fragments at their 5' ends. This suggests that the 5' extent of the deletion responsible for the creation of the 2.6-kilobase fragment lies beyond the region mapped by Lauer *et al.*²⁴ and therefore more than 25–30 kilobase upstream from the region of the normal α -globin genes. If this is the case, homozygosity for the 2.6-kilobase allele would result in defective embryonic haemoglobin synthesis. This lethal form of thalassaemia might go undetected due to the early time at which fetal loss would occur. The true incidence of the thalassaemia gene studied here among Mediterranean individuals is not known. As we identified this gene in 6 unrelated individuals with HbH disease among 12 cases initially analysed¹⁵, it cannot be exceedingly rare. We should anticipate, therefore, the discovery of fetal wastage due to homozygosity for this α -thalassaemia gene in populations in which it is prevalent rather than a hydrops fetalis phenotype¹, the consequence of deletion of the adult α -globin structural genes without simultaneous deletion of embryonic α -like genes.

We thank Dr David G. Nathan for his continued support and encouragement and J. Lauer and T. Maniatis for a preprint of ref. 24. S.H.O. is the recipient of a Research Career Development Award of the National Heart, Lung, and Blood Institute-NIH and a grant from the National Foundation March of Dimes. *Note added in proof:* The 3'-untranslated region of the α -thalassaemia gene is that of the normal α 1 gene. Therefore, the α -thalassaemia gene is derived from α 1 and the normal α -globin genes differ in sequence in that region.

Received 2 April; accepted 16 May 1980.

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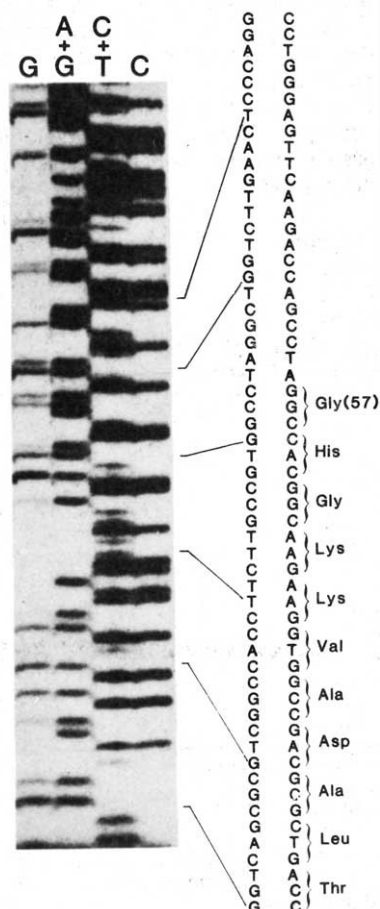
Errata

In the letter 'Mantle composition derived from the composition of lherozites' by S. Maaloe and R. Steel, *Nature* **285**, 321–322, the first line of paragraph 6 was omitted; it should read 'The composition of the mantle generating abyssal tholeiite'.

In the article 'Sequence of retrovirus provirus resembles that of bacterial transposable elements' by Shimotoono *et al.*, *Nature* **285**, 550–554, in Fig. 2 legend it should read 'Labelled termini are indicated by (–)' not (–) as shown. In Fig. 4 legend lines 21 and 22 should be omitted.

In the News and Views article 'Extra-pituitary actions of LHRH and its agonists' by Richard M. Sharpe, *Nature* **286**, 12–14, line 9 on page 14 should be replaced by 'LHRH has taught most of us to regard'

Fig. 4 Sequencing gel of the region containing the deletion in $\lambda\alpha$ T1(2.6). The 0.75-kilobase *Eco*RI/*Hind*III fragment of $\lambda\alpha$ T1(2.6) (Fig. 1) was 5' labelled at the *Hind*III site and subjected to the chemical degradation reactions of Maxam and Gilbert²⁵. The products were electrophoresed in a 0.38-mm thick 20% urea-acrylamide slab at 2,000 V for 24 h.



BOOK REVIEWS

Plants, politics and empires

A.H. Bunting

THE author offers this book as an account of "botanical imperialism" and a historical-anthropological analysis of the role of the Royal Botanic Gardens, Kew, in the expansion of the British Empire. Her purpose is to demonstrate that nineteenth-century Kew was a "control centre", the directors of which conspired with the Government to press the botanical resources of the Earth, and of Latin America in particular, into the service of British imperialism and the expansion of colonial rule, especially in Asia. Since she sees no redeeming facets in imperialism and colonialism, the tone of the book is overwhelmingly critical; and since both her objectives and her raw materials are restricted in scope, her presentation is out of balance. The broad sweep of Kew's immense contribution to pure and applied botany, the foundations of which were laid in the nineteenth century, hardly emerges.

About half of the book is devoted to setting the stage. An introduction, and a brief and superficial chapter on the political and economic history of the British Empire, are followed by an essay on the diffusion of domesticated plants around the world, which is offered as the primary reason for the growth of human population since 1492.

Two chapters on the growth and organization of science in Britain in the eighteenth and nineteenth centuries in general (including its social and personal links with government), and on the evolution of Kew and the overseas botanic gardens in particular, make much of class distinction and patronage; and so it is the more unfortunate that the Darwin-Wedgwood connection did not lead the author to ponder the relative roles of the Lunar and the Royal Societies. The special train which brought Wickham's 70,000 rubber seeds from Liverpool becomes an example of the power of authority serving imperial interest.

The next three chapters, on quinine, rubber and sisal, the core of the book, are followed by a final ten pages containing a curious miscellany — the Crystal Palace as a symbol of the common interests of feudal landowners and the new industrialists (because Paxton had learnt to build

Science and Colonial Expansion: The Role of the British Royal Botanic Gardens. By L.H. Brockway. Pp.215. (Academic: New York and London, 1979.) \$21, £12.

glasshouses at Chatsworth), leading on to the Palm House as a symbol of Kew's overseas role, a résumé of the history of British plant exploration and the growth of plantation industries, followed by some concluding remarks on the social function of science.

Taken as a whole, the book resembles, in structure, ideas and language, an unpolished and somewhat immature PhD thesis. It is a library study, supplemented by visits to Brazil and to Kew. Most of the references are to secondary sources, mostly from the social sciences, often written 100 years or more after the observations or events they review. A good deal of the biological comment is gauche — among much else, *Stylosanthes* is cited as a forage grass; the seed dispersal mechanism of *Hevea* cannot by itself explain the small density of trees of this genus in the Amazonian forest; *Ficus elastica* is not a vine; the significance of the monocarpic habit of *Agave* is misunderstood; and the nature of taxonomic work and the rules of priority in botanical nomenclature are misrepresented. If the biology is so unprofessional (and, moreover, written in a mixture of colloquial American and the metalanguage of an unfamiliar profession), what is the biological reader to think of the historical, economic and political material?

In fact the historical material about Kew, and the details of the transfers of plant materials in the eighteenth and nineteenth centuries, seem to be accurate enough. Much of it will be sufficiently unfamiliar to be interesting to non-specialists. The interpretation of history is something else. It makes much of smuggling and other knavish activities (though the circumstances in which Linnaeus' herbarium was transferred to Britain have escaped the author's censorious eye), and of the "conversion of knowledge to profit and power" by the British in support of

imperialism. Thus *Cinchona* plantations were established in the Nilgiris, with seeds and plants spirited out of the Andean countries, to aid control of malaria in the Indian Army in the aftermath of the Mutiny. Quinine then made it easier for the British to bring their families to live in India and so to widen the social gulf between British and Indians. Kew organized the smuggling of rubber out of Brazil — though J.W. Purseglove (*Tropical Crops, Dicotyledons* p.149. Longman: London, 1968) firmly rebuts this gloss on the events — for British military, industrial, commercial and other imperialist purposes. Kew is castigated for publishing, as scientific and technical information, secrets of the botany and processing of sisal which enabled Germany to obtain materials of a Mexican plant from a supplier in Florida in order to build a sisal industry in German East Africa which fell into Britain's lap when Tanganyika was mandated to her after the First World War.

The transfer of economic plant materials from one country to another is consistently treated as expropriation. While unique artefacts, or mineral resources, can be expropriated, genetic resources are by their nature renewable. Transferred plant materials can of course be used to produce improved varieties and build plantation industries which may bring far greater advantages to foreigners than to the people of their countries of origin. This is but one of the many harsh features of the uneven development of nations. Plant resources can be expropriated only where they are also eradicated in their country of origin, as the Dutch attempted to do with the clove tree early in the seventeenth century.

The cynic could comment here that many plant species and varieties are at present unlikely to be saved for posterity unless they are in part expropriated, since they are at severe and increasing risk in their native lands.

Many people today share the author's uncompromisingly critical attitude towards conquest, the oppression of man by man, imperialism in general (including neocolonialism and the multinational companies), and British imperialism in particular, and look askance at the



Kew Gardens in the 1850s, showing the newly constructed Palm House

arrogant self-righteousness of some nineteenth-century attitudes towards Britain's civilizing role among the lesser nations. We can well imagine that the destruction of indigenous weaving and metal industries in India, by imports from Britain, helped to provide a cheap labour force for plantation

industries in India (and later elsewhere in tropical Asia) when none was available in tropical America. Indeed this was advanced in the early 1830s as an argument for establishing a tea industry in India (*Tea Cultivation, India*. House of Commons Papers 63, 1839).

To most, however, this will seem an unduly eclectic view. Rubber provides a significant part of the economic basis for political independence in Malaysia and Indonesia; Indian quinine in its time saved many lives throughout the world; sisal has brought foreign exchange and some employment to Tanzania; and Kew continues to provide valued services, including third-country quarantine, for many developing countries.

Perhaps even more important are the personal and professional links, which include Kew and the Linnean Society, which hold together the large and active community of pure and applied botanists throughout the Commonwealth.

All in all, then, an unsatisfactory book; and in view of the great potential interest of its theme, a disappointing one.

A.H. Bunting, formerly Professor of Agricultural Botany, is Professor of Agricultural Development Overseas in the University of Reading, UK.

Krylov and the genesis of statistical physics

Michael Berry

Works on the Foundations of Statistical Physics. By N.S. Krylov. Pp.283. (Princeton University Press: Princeton and Guildford, UK, 1980.) Hardback \$19.50, £10.80; paperback \$7.50, £4.15.

RECENT YEARS have seen real progress towards answering fundamental questions in statistical mechanics, thanks largely to the Russian school led by Kolmogorov and Sinai. Their work was stimulated by the thorough and imaginative studies of N.S. Krylov, who died in 1947 at the tragically early age of 29. With the publication of this book, Krylov's works are at last available to English-speaking readers.

Krylov emphasized the importance of explaining how systems relax to equilibrium, and indeed regarded this as the central problem of statistical mechanics. He shows that a sufficient condition for relaxation is that the system's dynamics possess the property of mixing, which means that an initial patch of phase space (i.e. a group of systems) eventually spreads to cover the whole energetically available region in filamentary fashion (whilst preserving its area). Most previous work had concentrated on ergodicity, namely the property that a point in phase space (i.e. a single system) eventually passes arbitrarily close to every energetically available phase point. Ergodicity is a weaker property than mixing, and does not guarantee statistical

behaviour. Krylov traced the source of mixing (at least for systems of colliding hard spheres) to instability of trajectories, i.e. the exponential divergence of neighbouring orbits. He employed this idea in an approximate calculation of relaxation times. Subsequent research has abundantly confirmed that this type of instability is indeed the source of unpredictable behaviour in causal systems.

The book is not easy to read, because

Krylov's repetitious style of writing makes it hard to hold the thread of his complex argument. Nevertheless, this is an important document, which should be studied by anybody seriously interested in statistical mechanics. A valuable feature of this book is an appendix by Sinai, outlining recent developments of Krylov's ideas.

Michael Berry is Professor of Physics at the University of Bristol, UK.

Developments in plant molecular biology

Martin R. Hartley and R. John Ellis

Nucleic Acids in Plants. By T.C. Hall and J.W. Davies. Vol. I pp.261, Vol. II pp.237. (CRC: Boca Raton, Florida/Blackwell Scientific: Oxford, 1980.) Vol. I \$64.95, £48.75; Vol. II \$59.95, £44.75.

THESE two volumes represent the only comprehensive treatise so far published in the area of plant nucleic acids, and reflect the upsurge in interest and the exciting new developments that have recently taken place in plant molecular biology. The aim of the books is to provide a detailed and critical appraisal of selected topics which include many current research interests in plant nucleic acids, as well as some related aspects of protein synthesis and genetic manipulation. The title "*Nucleic Acids in Plants*" has allowed the inclusion of

contributions on viral, viroid and plasmid nucleic acids, which together with a chapter on gene manipulation by sexual and somatic cell techniques occupy Vol. II.

Volume I deals with nucleic acids found in 'healthy' plants, with articles on nuclear genome organization (Walbot and Goldberg), chloroplast DNA (Tewari), RNA polymerases (Becker), tRNAs (Weil), rRNA (Leaver) and mRNA (Hall). The styles of these chapters vary considerably, from epic literature reviews to more personal accounts. For example, Weil's 33-page article cites 432 references, while Tewari's 65-page contribution cites only 62 and relies largely on the author's own data. All of the articles are authoritative and are mostly easily readable, although by the very nature of some of the topics under discussion it has not always been possible to avoid a catalogue approach. Particularly impressive are Walbot and Goldberg's description of the intricacies of DNA reassociation kinetics in an intelligible fashion, and Becker's critical and reasoned account of plant RNA polymerases, in which he dispels many myths, including those about the action of plant growth substances at the transcriptional

level. Most articles contain fairly detailed sections on methodology, where advice is offered on the merits and limitations of different techniques. Although these sections are not sufficiently detailed to enable the reader to repeat experiments, they do provide valuable first-hand information of a kind which is not usually given in original papers or reviews, and therefore they should prove extremely useful for researchers entering the field. As is inevitable in such books, the reviews are out of date by up to two years, especially in the field of mRNA and chloroplast DNA where little of the recent cloning work is analysed.

Volume II contains chapters on plant DNA viruses (Hull), monopartite plant viruses (Zaitlin), multipartite and satellite plant viruses (Lane), the translation of plant viral RNA (Davies), viroids (Dickson), the crown gall phenomenon (Schell) and plant gene manipulation (Bingham). All are useful summaries of the state of the art at the time of writing, which varied from 1976 to 1978 judging from the references. Studies on plant viruses have not had the same impact on biology as have studies on bacteriophages and animal viruses, but several of the chapters stress their potential importance for improvements in agriculture. However the overall impression given is that plant virology is poorly developed compared to its bacterial and animal counterparts; these articles show that there is a long way to go before such studies lead to major improvements in agriculture by direct genetic manipulation. Thus the chapter by Hull is entitled "The DNA of Plant DNA Viruses" but most of the detailed information is confined to cauliflower mosaic DNA. That by Bingham discusses the various methods proposed for the genetic manipulation of crop plants from a plant breeder's point of view, and correctly stresses the severe nature of the problems to be overcome. His case is not helped by the fact that the work referred to on p.218 on the expression of foreign DNA in cultured plant cells has not stood the acid test of reproducibility in other laboratories. The contributions by Zaitlin, Lane and Davies contain much detailed information on the isolation, characterization, synthesis and translation of plant viral RNAs. These chapters will be especially useful to those entering the field.

The message stands out clearly that much more work of high quality is what plant virology needs before its full potential can be realized, and the latter volume should contribute towards this goal by informing and interesting young research workers. In this respect it is to be regretted that each slim book is so highly priced; 19p a page will deter many people from purchasing two useful volumes. □

Martin R. Hartley and R. John Ellis are Lecturer and Professor respectively in the Department of Biological Sciences at the University of Warwick, UK.

Catalytic chemistry of palladium

M.W. Roberts

Palladium Catalyzed Oxidation of Hydrocarbons. By P.M. Henry. Pp.407. (Reidel: Dordrecht, The Netherlands, Boston and London, 1980.) Dfl. 125, \$65.

IT IS perhaps surprising to someone not closely involved with the field that a book of over 400 pages can be entirely devoted to how palladium catalyses homogeneously the oxidation of hydrocarbons. However, a quick glance at this book left me in no doubt that it contains a wealth of interesting chemistry and that the idea is entirely justified. The book is aimed at three groups of people: (1) the industrialist interested in catalysis; (2) organic chemists interested in synthetic aspects; and (3) physical chemists interested in mechanistic aspects. The author is ideally suited to tackle such a task, being currently in a university post but having spent some 15 years in industry.

The book is divided into seven chapters. The author, however, having decided that there will be mainly two groups of readers — first, those largely interested in preparative chemistry, and second, those interested in both preparative and mechanistic aspects — has separated mechanistic discussions from the purely descriptive sections. Being mainly interested in heterogeneously catalysed reactions, I found this helpful.

In the first chapter we are led gently through a historical account, then some aspects of the basic inorganic chemistry of palladium in its various valence states — Pd(0), Pd(I) and Pd(III) — are described, followed by a discussion of the organometallic chemistry of palladium and finally catalysis. The two basic catalytic

mechanisms considered are: (1) oxidation-addition or its reverse, reduction-elimination; and (2) the insertion reaction or its reverse.

Chapter 2 deals exclusively with the oxidation of monoolefines leading to the formation of aldehydes and ketones, esters and ethers. Over half of the book is devoted to the first two chapters, while the remaining five deal with the reaction of dienes and polyenes, π -allyl complexes, reactions of acetylenes, reactions of aromatic compounds and what are termed "miscellaneous reactions".

An excellent bibliography of approximately 40 pages lists 1124 references largely taken from papers published during the past decade. Among the references there is a good sprinkling of patents reported, reflecting the author's links with the industrial application of palladium catalytic chemistry.

There is a great deal of information in this book which will cater for a wide cross-section of chemists. The detail is impressive and indicative of the author's close involvement with the subject. I found it perhaps unusual to have a blend of detailed preparative chemistry intertwined with mechanistic argument, but possibly physical chemists are unfamiliar with this approach! The author set out to "give a survey of both the range and utility of the (homogeneous) catalytic chemistry of palladium and to discuss the basic reaction paths...". This, I believe, he has accomplished satisfactorily. In addition there is much of benefit in this book for those interested in heterogeneous catalysis (and its possible relationship with homogeneous systems); however, what is difficult for the non-specialist to ascertain is how much of the mechanistic discussion is 'established fact' and how much 'interesting speculation'. □

M.W. Roberts is Professor of Physical Chemistry at University College, Cardiff, UK.

Alone; the story of South American mammals

R.J.G. Savage

Splendid Isolation: The Curious History of South American Mammals. By G.G. Simpson. Pp.256. (Yale University Press: New Haven and London, 1980.) \$17.50, £11.05.

FIFTY years ago Dr G. G. Simpson escaped death from revolutionaries in Argentina and undaunted carried on to lead a fossil-hunting expedition into distant Patagonia. He has returned many times to South America and has devoted a large pro-

portion of his long and very active scientific career to studying South American fossil mammals. In 1934 Simpson published a narrative of that first expedition; his deservedly popular book *Attending Marvels* (Macmillan: New York) vividly portrays the excitement of fossil hunting and the fascination of those bleak Patagonian landscapes. In *Splendid Isolation* he now addresses a wide readership with, as he subtitles the book, the curious history of South American mammals.

The book begins with chapters on the history of discovery from the time when Darwin found fossils there in 1832, on where and how fossil mammals occur, and on stratigraphical stages and mammal ages. For almost all of the past 60 million years the South American continent has been isolated without land connections

anywhere — neither to Antarctica or Africa, nor to Central or North America. A land link was only re-established within the past 5 million years. Thus throughout almost the entire Cenozoic era it was in splendid isolation, receiving only periodic mammal invasions by waif dispersal from some other continent(s). The history of the faunas is retailed in three phases: the earliest faunas of the Palaeocene and Eocene have just three basic stocks (the marsupials, xenarthrans and condylarths); in the second phase during the Oligocene and Miocene, we witness the addition of rodents and primates; and the third phase begins in the early Pliocene (as defined by Simpson) with the build-up to the great American interchange of mammal faunas north-south across the continents. The book finishes with a summary chapter and a note on the restoration of extinct animals.

The book is highly factual and, as we would expect of the doyen, highly accurate and up-to-date with the latest fossil discoveries. But the facts are too often dry and indigestible. Accounts of each family with descriptions of their dental characteristics do not make easy reading, the more so as few are adequately illustrated. Indeed the illustrations are almost totally limited to elemental reconstructions — all without scale. The Plio-Pleistocene and Recent faunas are very summarily treated; much of the space here is devoted to etymological discourse. Simpson's penchant for both logodaedaly and sesquipedality are evident throughout. There are no photographs, no skeletal reconstructions and only one palaeogeographical map — and that of the mid-Cretaceous. Palaeogeography is a subject to which the author has himself made signal contributions, yet it is all but ignored in this book; it merits space only in the last three pages. Considering the tremendous impact of plate tectonics theory over the past decade, much could have been said, if only in summary, about the continent's history and its relationships to adjacent regions during the past 80 million years. The author tells us his first and second phase colonizations were waif dispersals. The centre(s) of origin of waifs has been the subject of copious debate; Simpson, fairly but very briefly, disposes of the arguments on the grounds that we have too few facts to come to definitive conclusions. The third phase colonization is certainly better documented, but Simpson refuses to be drawn into the current debates on palaeogeographical models, dispersal or vicariance — the latter originating with a Venezuelan biologist. Many scientists feel such models are useful in that they offer a challenge to discover adequate evidence to test a hypothesis. With the immense knowledge and unique experience Simpson possesses, we would all appreciate and profit from having his palaeogeographical interpretation. For without this, the history is as Hamlet without the Prince of

Denmark. The task may be, to use Simpson's words, Herculean, but it is hardly Sisyphean.

There is no recent work with which *Splendid Isolation* can readily be compared. Patterson and Pascual's brilliant essay review of 1972, "The Fossil Mammal Fauna of South America" (In *Evolution, Mammals and Southern Continents*, edited by A. Keast *et al.* State University

of New York Press: Albany), is still indispensable to the serious student. Yet, while the balance of topics treated in *Splendid Isolation* is sometimes too light and sometimes too heavy, there is much a student will find interesting in this curious history. □

R.J.G. Savage is Reader in Vertebrate Palaeontology at the University of Bristol, UK.

Suspending time: a guide for biologists

David E. Pegg

Low Temperature Preservation in Medicine and Biology. Edited by M.J. Ashwood-Smith and J. Farrant. Pp.323. (Pitman Medical: Tunbridge Wells, UK/University Park Press: Baltimore, 1980.) £17.50, \$39.50.

WHEN Polge, Smith and Parkes made the discovery, in 1948, that glycerol would enable avian spermatozoa to survive freezing at -79°C , they could scarcely have anticipated the impact their observation was to have in medicine and biology. The special value of preserving viable cells in the deep-frozen state is that this essentially suspends time: chemical reactions are arrested and even the recrystallization of ice is prevented at the generally used storage temperature of -196°C , the boiling point of nitrogen. Then, only ionizing radiation can produce damage, but as Ashwood-Smith points out in this book, it would take 32,000 years to accumulate a dose of 600 rads! The cryobiologists' trick then is to cool cells to, and recover them from, -196°C without damage. Although the conditions necessary to achieve this differ widely from cell to cell, remarkable successes have been achieved: nowadays, innumerable scientists and technicians in fields as disparate as clinical blood banking, animal husbandry, immunology, reference culture collection of bacterial, fungal, animal and plant cells, and entomology use cryopreservation as a tool in their daily work, perhaps with little knowledge of the principles upon which the techniques are based. This book sets out to provide a guide for such workers.

Farrant opens the book with a delightfully clear elementary explanation of mechanisms of freezing injury and cryoprotection. Ashwood-Smith then describes current techniques for the preservation of tissue culture cells and some mammalian tissues, and Polge, truly one of the founders of cryobiology, contributes an excellent chapter on the development of techniques for the cryopreservation of spermatozoa — full of practical detail, but

also interestingly reflective on the way in which this field has developed. Whittingham gives excellent guidance from his wide personal experience of the low temperature preservation of early embryos of many mammals. The chapters on haematological applications, by Rowe and Schaefer, are essentially practical and detailed, while Knight provides an admirable critical account of work on the preservation of leukocytes, emphasizing the difficulties of obtaining valid measurements of post-thaw recovery. The chapters on protozoa and helminth parasites by James, on micro-organisms by Ashwood-Smith and on plant cells by Morris are all clear and packed with practical advice, while the contribution by Ring on the cryobiology of insects and their cells is particularly fascinating and goes beyond practical preservation to describe the fascinatingly varied adaptations found in insects that survive the extremely low temperatures occurring in Arctic regions. The book concludes with a chapter of general practical guidance.

Without doubt, this will prove to be a valuable text: it is not, however, as comprehensive as its title might suggest. Low temperatures, but without freezing, are widely used in medicine and biology for the short-term preservation of cells, tissues and organs, most importantly perhaps of blood cells for transfusion and of tissues and organs for transplantation. None of this is dealt with. Moreover the excellent introductory chapter could, I believe, usefully be supplemented by a more detailed discussion of theoretical cryobiology to help the reader digest the mass of 'cook-book' detail found in the subsequent chapters. Also, the balance of contributors seems a little strange in that it does not reflect the major contribution of workers in the USA during the past 20 years. Finally, the chapter on practical aspects would be much improved by the inclusion of greater detail of the actual equipment and procedures being discussed. (The thermocouple table on p.290 differs from the current BSI and NBS standards.) These are relatively small details, however, and the editors can be congratulated on a most useful compilation: perhaps they will consider widening the scope of future editions.

David E. Pegg is head of the MRC Medical Cryobiology Group in the Department of Surgery, University of Cambridge, UK.